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(54) Title: HIV-1 ENVELOPE MUTEINS LACKING HYPERVARIABLE DOMAINS

(57) Abstract

HIV-1 envelope muteins are provided comprising deletions within the hypervariable domains of the polypeptides. Methods of using these proteins in immunoassay and to elicit antibody production are also disclosed, as well as materials and methods useful for producing the muteins by recombinant DNA technology.

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HIV-1 ENVELOPE MUTEINS LACKING HYPERVARIABLE DOMAINS

Technical Field

The present invention is directed to novel analogs (muteins) of the envelope proteins from human immunodeficiency virus type 1 (HIV-1), methods of making the analogs, DNA sequences encoding the analogs, and methods of using the analogs, for example, in immunoassays and vaccine compositions.

Background

HIV-1, and a recently identified related virus named HIV-2, are the known causative agents of Acquired Immune Deficiency Syndrome (AIDS). Many isolates of HIV-1 have been identified and sequenced. A striking feature of these independent isolates is substantial genomic and amino acid variation, centered particularly in the envelope gene and proteins. This variation among HIV-1 isolates has important implications for both AIDS diagnostics and potential vaccines.

Starcich et al., (1986) Cell 45:637-648, reports on the genetic variation in five independent HIV-1 isolates, as well as variations in deduced amino acid sequences. Both conserved and variable regions were observed.

Coffin, (1986) Cell 46:1-4, is a review directed to the variation in the envelope of HIV-1, and the possible mechanism which brings about the variation. Coffin

hypothesized that the highly variable domains, termed "hypervariable" domains, are masking epitopes within the conserved domains from being available for neutralizing antibodies or cell-mediated immune responses. It is concluded that vaccination strategy for HIV-1 should be directed towards developing the ability to provoke an immune response directed against the conserved regions of the envelope despite the presence of masking variable domains.

Modrow et al., (1987) J. Virol. 61:570-578, is also directed to comparison of the amino acid sequences of various HIV-1 isolates. Computer analysis was employed to predict epitopes in the envelope protein, and it was found that the majority of predicted epitopes were located in the hypervariable regions. See, e.g., Figure 1 and Tables 1 & 2, incorporated herein by reference.

Hahn et al., (1986) Science 232:1548-1553, discloses the sequence variations in a series of HIV-1 isolates from a single individual, particularly in the envelope. See also Saag et al., (1988) Nature 334:440-444; Fisher et al., (1988) Nature 334:444-447.

Rusche et al., (1988) Proc. Natl. Acad. Sci. USA 85:3198-3202, discloses that a short peptide having the sequence of an HIV-1 isolate in the third hypervariable domain of the envelope protein was able to absorb isolate-specific neutralizing antibodies from antisera, and that another group was able to elicit isolate-specific neutralizing antibodies by immunization with a peptide from the same domain. It is suggested that since this neutralizing epitope is found in one of the hypervariable domains, that a possible vaccination strategy is to prepare a subunit antigen made up of the critical epitope from multiple isolates. See also Looney et al. (1988) Science 241:357-359.

Goudsmit et al., (1988) Proc. Natl. Acad. Sci. USA 85:4478-4482, reports on the neutralizing ability of antibodies raised in chimpanzees infected with various HIV-1 isolates, and a rabbit immunized with an envelope subunit. The epitope identified by Rusche et al. is reported to be an isolate-specific neutralizing epitope, and that it is immunodominant in HIV-1 chimpanzees.

Due in large part to the generally accepted view that attenuated or killed virus vaccines for AIDS are not feasible, the primary focus for the development of vaccines has been the subunit antigens. Several groups have suggested that short oligopeptides from the envelope domain would make suitable subunit antigens. Other groups are pursuing live recombinant virus vaccines, natural or recombinant viral polypeptide vaccines, or anti-idiotype vaccines. See, e.g., Koff et al., (1988) Science 241:426-432 (and references cited therein).

A continuing need exists to develop new and better HIV-1 envelope analogs, as well as polypeptides that avoid isolate-specific immune interactions for use in diagnostics and as potential vaccines.

Summary of the Invention

The present invention is directed to HIV-1 envelope analogs (muteins) comprising the constant domains of gp120env or gp160env, but lacking at least one epitope from a hypervariable domain. Despite the reports in the literature suggesting the importance of the epitopes in the hypervariable domains (e.g., immunodominant and/or neutralizing), it has surprisingly been discovered that the muteins of the present invention are useful as diagnostic reagents exhibiting at least as great or greater reactivity to antibodies raised against diverse isolates, and as antigens in raising nonisolate-specific antibodies upon immunization of a mammal.

In one embodiment, the present invention comprises an improved analog of HIV-1 gp120env or gp160env wherein the improvement comprises the deletion of at least one epitope within a hypervariable domain, while retaining the domains conserved among HIV-1 isolates.

In another embodiment, the present invention is directed to a polypeptide comprising epitopes bound by antibodies to HIV-1 gp120env or gp160env and an amino acid sequence according to the formula:

C1-V1-V2-C2-V3-C3-V4-C4-V5-C5

wherein:

C1 is an amino acid sequence substantially homologous to Ser29 through Cys130 of HIV-1 SF2;

C2 is an amino acid sequence substantially homologous to Cys199 through Leu291 of HIV-1 SF2;

C3 is an amino acid sequence substantially homologous to Ser366 through Cys387 of HIV-1 SF2;

C4 is an amino acid sequence substantially homologous to Cys415 through Gly456 of HIV-1 SF2;

C5 comprises an amino acid sequence substantially homologous to Phe466 through Arg509 or Leu855 of HIV-1 SF2;

V1 is an amino acid sequence of 0 to a maximum of about 30 residues;

V2 is an amino acid sequence of 0 to a maximum of about 50 residues;

V3 is an amino acid sequence of 0 to a maximum of about 90 residues;

V4 is an amino acid sequence of 0 to a maximum of about 30 residues; and

V5 is an amino acid sequence of 0 to a maximum of about 10 residues;

with the proviso that at least one of the V domains selected from the group consisting of V1, V2, V3, V4 and V5 contains no more than about one-third of the said maximum number of residues for the V domain.

Still another embodiment of the present invention is directed to a polypeptide analog of HIV-1 gp120env or gp160env comprising (a) about 300 to about 850 amino acid residues in length; (b) constant domains, in the N-terminal to the C-terminal direction, Ser29-Cys130 of HIV-1 SF2 (C1), Cys199-Leu291 of HIV-1 SF2 (C2), Ser366-Cys387 (C3), Cys415-Gly456 (C4) of HIV-1 SF2, and Phe466-Arg509 or Phe466-Leu855 of HIV-1 SF2 (C5), or domains substantially homologous to said C1, C2, C3, C4 or C5; and (c) the intervening domains, if any, located between said constant domains comprising sequences found between substantially homologous constant domains in native HIV-1 gp120env, with the proviso that at least one of said intervening domains between said constant domains is either missing or missing an epitope.

The present invention is also directed to an immunoassay for the detection of antibodies to HIV-1 comprising: (a) providing a liquid sample to be tested for the presence of anti-HIV-1 antibodies; (b) contacting said sample with a polypeptide as described above under conditions whereby any anti-HIV-1 antibodies present in said sample may bind to an epitope to provide sample-contacted polypeptide; and (c) detecting any antibody bound to said sample-contacted polypeptide.

In yet another embodiment, the present invention is directed to a method of selectively raising antibodies in a mammal to epitopes in the constant domains of human immunodeficiency virus type 1 (HIV-1) gp120env or gp160env comprising administering to said mammal a polypeptide as described above, whereby antibodies to said polypeptide are produced in response to said administration.

The present invention is also directed to a composition useful in such method comprising the polypeptide described above in combination with a pharmaceutically acceptable carrier.

The present invention is also directed in another embodiment to a DNA sequence encoding the above polypeptide, as well as a cellular host comprising the DNA sequence under the control of transcriptional and translational control sequences whereby the polypeptide encoded by the DNA sequence is expressed by the cellular host. The present invention is also directed to methods of producing the above polypeptide by growing a culture of the above cellular host under conditions whereby the polypeptide encoded by the above DNA sequence is expressed, and recovering the polypeptide from the culture.

These and other embodiments of the present invention will be apparent to those of ordinary skill in the art.

Brief Description of the Figures

Figure 1 is a schematic diagram of the wild-type gp120env gene from HIV-SF2.

Figure 2 shows the alignment of amino acid sequences for various HIV-1 isolates, including SF2, with the constant and variable domains indicated. Potential N-linked glycosylation sites, for the HXB2 sequence only, are indicated by "[ ]"; cysteine residues have "*" above them.

Figure 3 is a restriction map of mammalian expression vector pSV7dARV120tpa.

Figure 4 is a restriction map of mammalian expression vector pCMV6ARV120tpa.

Figure 5 is a restriction map of yeast expression vector pHL15.

Figure 6 contains graphs showing the result of an ELISA testing the reactivity of HIV-1 envelope muteins of the present invention and controls against a North American serum panel.

Figure 7 contains graphs showing the result of an ELISA testing the reactivity of HIV-1 envelope muteins of the present invention and controls against an African serum panel.

Figure 8 shows the results of an ELISA using sera from guinea pigs immunized with envelope muteins according to the present invention or various controls.

Figure 9 shows the results of an ELISA using sera from goats immunized with envelope muteins according to the present invention or various controls.

Detailed Description

HIV-1 is a known virus, of which many isolates have been observed. See, e.g., Levy et al., (1984) Science 225:840; Barre-Sinoussi et al., (1983) Science 220:868; Popovic et al., (1984) Science 224:497; AIDS: Papers from Science, 1982-1985 (R. Kulstad ed. 1986); Current Topics in AIDS (M. Gottlieb et al. eds. 1987). The envelope gene of HIV-1 produces a precursor glycoprotein of approximately 160 mw, referred to as gp160 or gp160env. The precursor is cleaved during expression to provided gp120 (gp120env), the major exterior envelope glycoprotein, and gp41 (gp41env), the transmembrane protein. Various HIV-1 isolates have been reported in the literature, including, BH10, PV22, BRU, HXB2, WMJ1, WMJ2, WMJ3, CDC4, HTLV-IIIRF, HTLV-IIIB, Z3, Z6, Z321, MAL, NY5, ELI, JY1, LAV1A, and HAT3. Of particular interest to the present invention is HIV-1 isolate SF2, originally designated ARV2. See, e.g., Levy, U.S. Patent No.

4,716,102; Levy et al., (1984) Science 225:84; EPO Pub. No. 181,150.

The conserved and hypervariable domains of HIV-1 envelope proteins have been described previously. See, e.g., Modrow et al., supra. Figure 1 herein is a schematic diagram showing the location of the five hypervariable regions (V1, V2, V3, V4 and V5) and the five constant regions (C1, C2, C3, C4 and C5) in the envelope gene from HIV-1 SF2. Various deletions encompassing part or all of a hypervariable domain are also shown (D1, D2, D3, D4 and D5). Two regions of predominantly hydrophobic amino acids are denoted by dark shading, where the transmembrane anchor has been labeled TM. The signal sequence is highlighted at the N-terminus, as well as the processing site separating gp120env (positions 1-1527) from gp41env (positions 1528-2565).

Hypervariable domains are characterized by a substantial lack of homology (e.g., as low as 10%) among independent HIV-1 isolates. Furthermore, there is a substantial variation in length among the hypervariable domains from various isolates due to the prevalence of insertion and deletion mutations. Thus, these regions cannot be characterized from one isolate to the next by having any substantial degree of amino acid sequence homology, and can only be assigned an approximate length. The primary characterization of hypervariable domains is their location within the envelope glycoprotein and their presumed tertiary structure (i.e., loops). The conserved or constant domains, as well as all 18 cysteines in the envelope, are highly conserved. Corresponding hypervariable domains are found to be located identically relative to surrounding constant domains and cysteines from one isolate to the next. Furthermore, the tertiary structure of the hypervariable domain appears to be highly conserved; e.g., two nonhomologous hypervariable domains

from different HIV-1 isolates will usually both exhibit the same three-dimensional conformation, such as an exposed loop. Thus, hypervariable domains from new HIV-1 isolates can be readily identified by sequencing the new isolates and comparing the sequence to known HIV-1 sequences so that the conserved domains and cysteines are aligned. See, e.g., Modrow et al., supra and Figure 2 herein.

The location of the various domains, both constant and hypervariable, will be described hereinafter with reference to the sequence and numbering of the HIV-1 SF2 isolate. It is to be understood that this is for convenience only; the invention is not limited to analogs containing only HIV-1 SF2 sequences, but also encompasses analogs employing corresponding domains or sequences from other isolates. A domain from another HIV-1 isolate envelope protein can be easily identified as corresponding to an SF2 sequence by those of ordinary skill in the art by alignment of the conserved domains and the 18 cysteines of both isolates. Such an alignment is shown in Figure 2, where the corresponding sequences of 15 HIV-1 isolates are aligned. The cysteine residues are marked with an asterisk, and potential N-linked glycosylation sites for the HXB2 isolate are indicated by "[ ]". The cleavage site for the signal peptide and for the mature gp120env and gp41env proteins are shown, along with the constant domains, C1-C5. Specific deletions, D1-D5, are also shown in the figure.

The constant domains of the SF2 isolate are as follows: C1, Ser29-Cys130; C2, Cys199-Leu291; C3, Ser366-Cys387; C4, Cys415-Gly456; and C5, which is Phe466-Arg509 for gp120env analogs, or Phe466-Leu855 for gp160env analogs. Since these domains are highly conserved among HIV-1 isolates, the corresponding sequences from isolates other than SF2 will be substantially homologous to these

SF2 domains; i.e., a minimum of about 70-75% amino acid sequence homology, with certain highly conserved domains exhibiting a minimum of about 80%, or even 85-90% homology. The differences in amino acid sequences found in these conserved domains are generally attributable to point mutations in the nucleic acid sequence, as opposed to the deletion and insertion mutations which typify the differences in hypervariable domains.

As shown in Figures 1 and 2, the hypervariable domains lie between the conserved domains, C1-C5. Not all of the amino acids in these intervening domains, however, comprise hypervariable regions. Indeed, due to the heterogeneity among HIV isolates in the hypervariable domain, it is not feasible to generally define precise limits of the hypervariable domain. Thus, for the convenience of describing the present invention, the domains between the constant domains will be referred to as "variable" or "intervening" domains, and it will be understood that they may comprise both hypervariable regions as well as less variable, yet not highly conserved, sequences. Thus, the analogs of the present invention can be described schematically according to the following formula:

C1-V1-V2-C2-V3-C3-V4-C4-V5-C5 (I)

C1-C5 and V1-V5 in formula I are defined as described above in the Summary of the Invention. V1-V5 consist of the variable or intervening domains which contain hypervariable regions. The polypeptides of the present invention will contain a deletion in at least one of these variable domains when compared to a native gp120env or gp160env. The deletions will typically be at least about one-third of the variable domain, and oftentimes will consist of a deletion of the entire variable domain.

Furthermore, it is also preferred to delete sequences from more than one of the variable domains, including the deletion of all five of the variable domains in their entirety. Thus, in accordance with the present invention, any of the variable domains, V1-V5, can be anywhere from zero to a maximum number of amino acids in length, the maximum being an approximation of the longest of such domains found in HIV-1 isolates. At least one of the V domains, however, will have deleted therefrom at least one-third of that maximum number of amino acids, or one-third of the length of the corresponding domain from the homologous HIV-1 isolate.

Since the variable or intervening domains of formula I can comprise more than the true hypervariable domains, a selected Vn domain can be expressed by the following formula:

$$\text{Sn-HVn-Sn}' \qquad \text{(II)}$$

wherein n is 1, 2, 3, 4 or 5 (as in V1, V2, etc.), HVn is a hypervariable domain of x amino acid residues in length, and Sn and Sn′ are nonhypervariable sequences flanking HV in the Vn domain, the flanking sequences being y and y′ residues in length, respectively. In the native protein, the sum of x, y and y′ equals the maximum number of residues for Vn in a chosen isolate, and y and/or y′ may be zero. As for the analogs of the present invention, the sum of x, y and y′ will be anywhere from zero to the maximum number. The actual values for x, y and y′ for a particular Vn can be determined by sequence comparison of a representative number of HIV-1 isolates. See, e.g., Figure 2 and Modrow et al., supra.

The following are the intervening domains of the SF2 isolate, which can be used for comparison purposes to determine the corresponding intervening domains in other

HIV-1 isolates. See, e.g., Figure 2. V1 of SF2 is about 24 amino acids in length, encompassing Thr131 through Asn154 and, optionally, Cys155. This C-terminal cysteine is one of the 18 highly conserved cysteine residues found in gp120env. The corresponding domains from independent HIV-1 isolates sequenced to date appears to range from about 22 to about 31 amino acid residues in length. V2 of SF2 spans from Ser156 through Ser198. The corresponding domain in other HIV-1 isolates sequenced to date appears to range from about 39 to about 52 amino acid residues in length. V3 of SF2 spans Asn292 through Glu365, and the corresponding domains in other HIV-1 isolates sequenced to date appears to range from about 88 to about 90 amino acid residues in length. V4 of SF2 spans from Asn388 through Pro414, and the corresponding domains from other HIV-1 isolates appear to range from about 28 to about 33 residues in length. V5 of SF2 spans from Thr457 through Thr463, and corresponding domains from other HIV-1 isolates appear to range from about 10 to about 11 residues in length.

In selecting the areas of the intervening domains for deletions, it is preferred to either select those portions showing hypervariability, or areas known to encode epitopes which elicit a significant immune response in vivo. Examples of such epitopes in variable regions of the SF2 isolate include, without limitation, residues 137-158 (V1), residues 189-209 (V2), residues 300-327 (V3), residues 367-384 (V3), and residues 404-420 (V4). Corresponding epitopes from other isolates are known. Modrow et al., supra. Examples of preferred deletions for SF2, D1-D5, are shown in Figures 1 and 2: Thr131-Asn154 (D1), Ser156-His198 (D2), Thr300-His332 (D3), Asn388-Pro414 (D4), and Thr457-Thr463 (D5). The polypeptides of the present invention may also have deletions from more than one variable domain, for example, V1 and V2; V1, V2 and

V3; V1, V2 and V5; V3, V4 and V5; or V1, V2, V3, V4, and V5.

In a preferred embodiment, the HIV-1 envelope analogs of the present invention have deleted therefrom an epitope found in the intervening domains which is bound by antibodies produced by a mammal infected or immunized with the particular HIV-1 isolate, or its native envelope protein. It is particularly preferred that the deleted epitopes are those which produce isolate-specific immunodominant responses in a mammal. While applicants do not wish to be bound by this theory, it is believed that the deletion of these variable epitopes unmasks epitopes in the conserved domains which then become visible to the immune system by virtue of the absence of the epitopes from the variable domains.

In general, the amino acid sequence according to formula I will range from about 300 to about 850 amino acid residues in length, the actual length not being critical. The sequence of formula I can be contained within a larger polypeptide, for example a fusion protein. Such fusion proteins can include, for example, a fusion between the N-terminal sequence of superoxide dismutase (human or yeast) or beta-galactosidase, where these non-HIV-1 sequences are fused to the N-terminal of the C1 domain. Alternatively, non-HIV-1 sequences could also be fused to the C-terminal of the C5 domain. The C1 domain may also be fused to a signal peptide (e.g., yeast alpha factor, or tpa signal) to provide for secretion of the HIV-1 envelope analog from a cellular host expressing the analog.

The HIV-1 envelope muteins of the present invention can be produced by any suitable method, such as direct peptide synthesis or recombinant DNA expression. The preferred method is to prepare the polypeptides by recombinant DNA techniques.

The methodology for preparing such recombinant polypeptides is within the skill of the art, and the techniques are fully explained in the literature. See, e.g., Maniatis et al., Molecular Cloning: A Laboratory Manual (1982); DNA Cloning: A Practical Approach, Volumes I & III (D.N. Glover, ed., 1985); Oligonucleotide Synthesis: A Practical Approach (M.J. Gate, ed., 1984); Perbal, A Practical Guide to Molecular Cloning (1984). The production of recombinant HIV-1 polypeptides is known in the art. See, e.g., Luciw et al., (1984) Nature 312:760; Sanchez-Pescador et al., (1985) Science 227:484; Hahn et al., (1984) Nature 312:167; Alizon et al., (1984) Nature 312:757; Ratner et al., (1985) Nature 313:636; Muesing et al., (1985) Nature 313:450; Wain-Hobson et al., (1985) Cell 40:9; EPO Pub. Nos. 181,150; 187,041; 227,169; 230,222; and PCT Pub. Nos. WO87/02038; WO87/02989; WO87/04459; WO87/04728. Methods of recombinant expression are also disclosed in commonly owned U.S. patent application Serial No. 138,894, filed 24 December 1987, entitled "Human Immunodeficiency Virus (HIV) Nucleotide Sequences, Recombinant Polypeptides, and Applications Thereof," the disclosure of which is incorporated herein by reference.

To prepare the polypeptides of the present invention by recombinant methods, a DNA coding sequence for the polypeptides must be provided. Such a coding sequence is a DNA sequence that can be transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by and include the translation start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. DNA sequences encoding the polypeptides of the present invention can be prepared either by constructing a synthetic gene from overlapping oligonucleotides, or by site-directed mutagenesis of a sequence encoding native

HIV-1 envelope. See, e.g., Zoller, & Smith (1983) Meth. Enzymol. 100:468-500.

DNA coding sequences for the polypeptides of the present invention can be maintained on a replicon, which is any genetic element (e.g, plasmid, cosmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control. Vectors are replicons such as a plasmid, phage, or cosmid to which a DNA coding sequence may be attached so as to bring about the replication of the attached segment *in vivo*.

A host cell is then transformed with a DNA construct containing the coding sequence under the control of appropriate regulatory sequences in order to bring about the expression of the DNA coding sequence into the desired polypeptide. Cellular hosts can include, but are not limited to, bacteria (e.g., *E. coli*, *Bacillus subtilis*, *B. amyloliquefaciens*, *Salmonella typhimurium*, *Klebsiella pneumoniae* or *Erwinia amylouora*), yeast (e.g., *Saccharomyces cerevisiae*, *S. carlsbergensis*, *S. kluyveri*, *Kluyveromyces lactis*, Pichia, or Schizosaccharomyces), mammalian cells (e.g., CHO cells, COS cells, 293 cells or Xenopus oocytes), and insect cells (e.g., Drosophila embryos or *Spodoptera frugiperda*). The transformed cellular hosts may contain the DNA constructs encoding the polypeptides of the present invention either on an extrachromosomal element, or integrated into the chromosome.

Typically, the DNA coding sequence is placed into an expression cassette which comprises the DNA coding sequence flanked by appropriate regulatory sequences that control transcription initiation and termination within the cellular host. Preferably, the expression cassette contains convenient restriction sites at either end to

permit convenient cloning of the cassette into an appropriate vector for transformation of the cellular host.

Transcription initiation and termination sequences recognized by the cellular host are DNA regulatory regions which flank a coding sequence are responsible for the transcription of an mRNA homologous to the coding sequence which can be translated into the desired polypeptide. Transcription initiation sequences include host promoter sequences, which are DNA regulatory sequences capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. A coding sequence is "under the control" of transcription initiation and termination sequences when RNA polymerase binds the transcription initiation sequences and transcribes the coding sequence into mRNA terminating at the transcription termination sequence, and the mRNA is then translated into the polypeptide encoded by the coding sequence (i.e., expression).

Cellular hosts transformed with appropriate DNA constructs for the expression of polypeptides of the present invention are typically grown in a clonal population under appropriate conditions which bring about the expression of the DNA coding sequence of interest. The appropriate growth conditions will depend upon the cellular host and the transcriptional and translational regulatory sequences employed. Upon expression, the recombinant polypeptide is recovered from the culture by any appropriate method, e.g., gel chromatography, immunoabsorption, or gel electrophoresis.

Polypeptides of the present invention can be used as reagents in immunoassays for detecting the presence in a sample of either anti-HIV-1 antibodies or viral antigen. In an immunoassay for viral antigen, for example, polypeptides according to the present invention

can be labeled and used as a competing antigen in a standard competitive ELISA or radioimmunoassay (RIA).

Configurations for immunoassays for antibodies to HIV-1 envelope vary widely, and the present invention contemplates the use of the polypeptides described herein in any such format. Such formats are well known in the art. See, e.g., Immunoassay: A Practical Guide (P.W. Chan & M.T. Perlstein, eds., 1987); McDougal et al., (1985) J. Immunol. Meth. 76:171-183; U.S. Pat. Nos. 4,629,783; 4,281,061; 4,520,113; 4,591,552; 4,134,792 (incorporated by reference herein). Whether the assay format is homogeneous or heterogeneous, and the measurement method is direct or indirect (e.g., competition), all such immunoassays have three common steps. First, a liquid sample suspected of containing the antibodies is provided. This sample is then contacted with polypeptides according to the present invention under conditions which will allow any antibodies having an epitope on the polypeptides to become bound thereto. Finally, there is a detecting step wherein it is determined whether or not any antibody bound to the polypeptide.

A preferred format for an anti-HIV-1 antibody assay is a heterogeneous immunoassay in which the polypeptides of the present invention are immobilized on, for example, a solid support. The selection of the appropriate solid support for immobilization of the polypeptide is conventional and within the skill of the art. In a typical assay, the sample suspected of containing the antibodies is contacted with the immobilized polypeptide and allowed to incubate under the appropriate conditions. The immobilized polypeptide is then separated from the sample and washed to remove any unbound antibody. The detecting step can constitute, for example, contacting the washed, immobilized polypeptide with an antibody that will recognize an epitope located on the anti-HIV-1 antibodies

(i.e., anti-xenogenic), this second antibody being appropriately labeled for detection (e.g., radiolabeled, enzyme conjugated, avidin/biotin). After an appropriate incubation and washing step, the immobilized polypeptide is then assayed for the presence of the labeled second antibody. Alternatively, a competition immunoassay can be used where a labeled reference antibody to the immobilized polypeptide is incubated along with the sample suspected of containing the anti-HIV-1 antibodies, and the presence of such antibodies are determined by measuring a reduction or inhibition of binding of the labeled reference antibody to the immobilized polypeptide. See, e.g., PCT Pub. No. WO87/07957.

Kits suitable for immunodiagnosis and containing the appropriate labeled reagents are constructed by packaging the appropriate materials, including the polypeptides of the present invention and any antibodies used in the assay, in suitable containers along with remaining reagents and materials required by the assay format; e.g., incubation media, wash media, and means for measuring the presence of the analyte (e.g., enzyme-labeled antibodies and enzyme substrate). Other labels useful in the practice of immunoassays according to the present invention include radioisotopes and fluorescing compounds.

Polypeptides according to the present invention can be employed to selectively raise antibodies in a mammal to epitopes found in the constant domains of gp120env or gp160env. For example, parenterally administering polypeptides of the present invention to a mammal will cause an immune reaction in the animal, thereby producing antibodies to epitopes found in the conserved domains. Such antibodies can be recovered to make polyclonal antiserum, or antibody-producing cells recovered for fusion (or another immortalization technique) to produce

monoclonal antibody-producing cell lines. See, e.g., M. Schreier et al., Hybridoma Techniques (1980); Hammerling et al., Monoclonal Antibodies and T Cell Hybridomas (1981); Kennett et al., Monoclonal Antibodies (1980); U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887. Antiserum produced by the above method will be advantageous in that it will possess a high titer of antibodies which are reactive to all or most HIV-1 isolates. In a similar manner, the screening of hybridomas for non-isolate-specific antibodies will be facilitated by the elimination of some or all of the immunodominant epitopes of the hypervariable regions.

To generate such an antibody response, polypeptides of the present invention are typically formulated with a pharmaceutically acceptable carrier for parenteral administration. The formulation of such compositions, including the concentration of the polypeptide and the selection of the vehicle and other components, is within the skill of the art.

A pharmaceutically acceptable vehicle, suitable for parenteral injection, is usually nontoxic and nontherapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and Hank's solution. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used. Parenteral vehicles may also take the form of suspensions containing viscosity-enhancing agents, such as sodium carboxymethylcellulose, sorbitol, or dextran. The vehicle will also usually contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and tris buffer, while examples of preservatives include thimerosal, m- or c-cresol, formalin and benzyl alcohol. The muteins of the present invention may also be formulated into liposomes for parenteral

administration. Standard formulations will either be liquid injectables or solids which can be taken up in a suitable liquid as a suspension or solution for injection. Thus, in a nonliquid formulation, the vehicle may comprise dextrose, human serum albumin, preservatives, etc., to which sterile water or saline could be added prior to administration.

Various adjuvants are known in the art which can also be employed in the vaccine formulations of the present invention; e.g., Freund's adjuvant, avridine, aluminum salts [$Al(OH)_3AlPO_4$, $Al_2(SO_4)_8$], $Ca_3(PO_4)_2$, saponin, DDA, Plusonics, oil-in-water emulsions (containing, e.g., avridine, dextran sulfate or vitamin E), water-in-oil emulsions (containing, e.g., polysorbate 8), and muramyl peptides (e.g., di- and tripeptides in carriers such as oil-water emulsions or liposomes). The selection of the appropriate adjuvant and its concentration in the vaccine composition is within the skill of the art.

Many protocols for administering the vaccine composition of the present invention to animals are within the skill of the art. The preferred route of administration is parenteral, particularly intramuscular, although administration may also be intravenous. The concentration of the polypeptide antigen in the vaccine composition is selected so that an effective dose is presented to the host mammal (e.g., primate) to elicit antibodies to the polypeptide's epitopes. Within wide limits, the dosage is not believed to be critical. Typically, the vaccine composition is administered in a manner which will deliver between about 1 to about 1,000 ug of the polypeptide antigen in a convenient volume of vehicle (e.g., about 1-10 ml). Preferably, the dosage in a single immunization will deliver from about 1 to about 500 ug of polypeptide antigen, more preferably about 5-10 to about 100-200 ug (e.g., 10-100 ug). It may also be preferred, although

optional, to administer a second, booster immunization to the mammal several weeks to several months after the initial immunization. It may be helpful to readminister a booster immunization to the mammal once every several years to maintain high antibody titer.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the claims in any way.

EXAMPLES

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The following example describes the construction of DNA sequences encoding HIV-1 SF2 envelope muteins according to the present invention. The hypervariable regions and the corresponding deletions are shown below in Table 1.

Table 1
Hypervariable Regions and Deletion Mutants

Hypervariable Regions		Corresponding Deletion Mutant(s)	
V1	131 through 154	D1	131 through 154
V2	156 through 198	D2	156 through 198
V3	292 through 365	D3	300 through 332
V4	388 through 414	D4	388 through 414
V5	456 through 465	D5	457 through 463

DNA encoding sequences for muteins of the present invention were prepared by mutagenesis of a gp120env SF2 gene. Mutagenesis was achieved employing the procedure described by Zoller and Smith, (1983) Meth.

Enzymol. 100:468-500, modified as described below. Synthetic DNA mutagenesis and sequencing primers (Table 2) were prepared by automated oligonucleotide synthesis on a silica support as described by Urdea et al., (1983) Proc. Natl. Acad. Sci. USA 80:7461-7465, using N,N-diisopropyl phosphoramidites. Sequencing primers were designed for sequencing by the dideoxynucleotide chain termination method in bacteriophage M13. Sanger et al., (1977) Proc. Natl. Acad. Sci. USA 74:5463. The sequencing primers were designed to be complementary to M13mp8 recombinant templates, of 18 bases in length, and to anneal at a position at least 50 bases away from the mutation locus. Table 2 indicates the oligomers employed for mutagenesis screening and sequencing..

The mutagenesis template contained the entire plasmid pSV7dARV120tPA, which contains the SF2 gp120env gene coupled to the tPA signal sequence (described below). The plasmid was linearized at its unique PstI site and cloned into the unique PstI site of M13mp8.

Table 2

Mutagenesis Primers	
D1	5′ CCACTCTGTGTTACTTTAAATTGCTGCTCTTTCAATATCACCACAAGC 3′
D2	5′ ATAAAAGGAGAAATAAAAAACTGCTGTAACAGATCAGTCATTACACAG 3′
D3	5′ AATGAATCTGTAGCAATTAACTGTTGTAACATTAGTAGAGCACAATGG 3′
D4	5′ GGGGAATTTTTCTACTGTTGTAGAATAAAACAAATTATAAACATGTGG 3′
D5	5′ CTGCTATTAACAAGAGATGGTGGTGAGGTCTTCAGACCTGGAGGAGGA 3′
D1+D2	5′ CCACTCTGTGTTACTTTAAATTGCTGCTGTAACAGATCAGTCATTACA 3′

DNA Sequencing Primers

D1,D2,D1+D2	5′ TAATCAGTTTATGGGATC 3′
D3	5′ CTGTTAAATGGCAGTCTA 3′
D4,D5	5′ CAATCCTCAGGAGGGGAC 3′

Screening Probes

D1	5′ TTAAATTGCTGCTCTTTC 3′
D2	5′ AAAAACTGCTGTAACAGA 3′
D3	5′ AATTAACTGTTGTAACATTA 3′
D4	5′ GGGGAATTTTTCTACTGTTGTAGAATAAAACAAATTATAAACATGTGG 3′
D5	5′ ATGGTGGTGAGGTCTT 3′
D1+D2	5′ CCACTCTGTGTTACTTTAAATTGCTGCTGTAACAGATCAGTCATTACA 3′

Mutagenesis of M13/pSV7dARV120tPA recombinants was performed using purified templates by annealing and extending the appropriate primer with the Klenow fragment of DNA polymerase I. Individual deletion mutants D1, D2, D3 and D5 were obtained utilizing the appropriate mutagenesis primer in a single round of mutagenesis (see Table 2). Deletion mutant D4 was obtained utilizing the appropriate mutagenesis primer with the annealing and elongation reactions performed at 37°C and in the presence of 125 ug/ml gene 32 protein. Combination deletion mutant D1+D2 was obtained utilizing the appropriate mutagenesis primer using a template derived from deletion mutant D1.

Following transfection of JM101 cells (Zoller and Smith, supra), plaques were grown at a density of 200-1,000/plate and lifted onto filters and screened by hybridization with the appropriate mutagenesis primer or probe (see Table 2).

The DNA sequence of putative positive clones was determined using suitable primers and template preparations. Once the mutagenized locus and flanking segments (i.e., at least 50 bases) were confirmed by DNA sequence analysis, replicative form (RF) DNAs were digested by PstI restriction endonuclease and the entire mutagenized mammalian expression vector pSV7dARV120tPA containing the deletion was recovered. The vector provides an SV40 early promoter and enhancer for expression of SF2 gp120env gene, SV40 polyadenylation site, and an SV40 origin of replication for use of the vector in COS cells.

Following recovery of pSV7dARV120tpa plasmids for wild-type gp120 or for a deletion mutant, the plasmid was digested with SalI to excise the complete gene spliced to the human tPA 5′ untranslated sequences and signal sequences. The SalI fragment was subcloned into the unique SalI cloning site of the mammalian cell expression vector pCMV6a. The resulting plasmid was screened to verify the correct orientation of the gene with respect to the promoter and polyadenylation signals, and this plasmid was named pCMV6ARV120tpa for the wild-type gp120 sequences. In cases where the pSV7d vector was not recovered directly as a plasmid, the SalI fragment containing the gene was subcloned from the M13 mutagenesis template clone into pCMV6a.

Combination deletion mutant D4-D5 was obtained utilizing the appropriate D5 mutagenesis primer using a template derived from deletion mutant D4.

Combination deletion mutant pSV7d120D3-D4-D5 was obtained by subcloning the region containing D4-D5 by digestion of M13pSV7d120D4-D5 with MstII and HindIII and insertion of this 334 bp fragment into MstII and HindIII digested pSV7d120D3. The SalI fragment from pSV7d120D3-D4-D5 containing D3-D4-D5 was subcloned into the SalI site of pCMV6a to create pCMV6a120D3-D4-D5.

Combination deletion mutant D1-D2-D5 was obtained by subcloning the region containing D1-D2 by digestion of pCMV6a120D1-D2 with NheI and BglII and insertion of this 540 bp fragment into NheI and BglII digested pCMV6a120D5.

Combination deletion mutant D1-D2-D3-D4-D5 was obtained by subcloning the fragment containing the D1-D2 region by digestion of M13pSV7d120D1-D2 with NheI and BglII and insertion of this 539 bp fragment into NheI and BglII digested pCMV6a120D3-D4-D5.

Expression vector pCMV6a can be regenerated by excising the coding sequence for gp120 from pCMV6ARV120tpa with SalI. The mutein coding sequences described above can all be constructed from the wild-type gp120 coding sequence in pCMV6ARV120tpa as described for pSV7dARV120tpa. Table 3 sets forth the names and deletions of the various M13-pSV7d- and pCMV6a-based vectors made according to the above protocol.

Table 3

Gene Version	M13 Vector	pSV7d Vector	pCMV6a Vector
Wild-type	M13pSV7d120	pSV7dARV120tpa	pCMV6ARV120tpa
D(1+2)	M13pSV7d120D1+2	not made	pCMV6a120D1+2
D1	M13pSV7d120D3	pSV7d120D1	pCMV6a120D1
D2	M13pSV7d120D2	pSV7d120D2	pCMV6a120D2
D3	M13pSV7d120D3	pSV7d120D3	pCMV6a120D3
D4	M13pSV7d120D4	not made	pCMV6a120D4
D6	M13pSV7d120D5	not made	pCMV6a120D5
D3+D4	M13pSV7d120D3+D4	not made	not made
D4+D5	M13pSV7d120D4+D	pSV7d120D4+D5	not made
D3+D4+D5	not made	pSV7d120D3+D4 +D5	pCMV6a120D3+D4 +D5
D(1-5)	not made	not made	pCMV6a120D1-5
D(1+2+5)	not made	not made	pCMV6a120D(1+2 +5)

pSV7dARV120tPA (Figure 3) was constructed as follows. An env gene was modified by in vitro mutagenesis to eliminate any potential transmembrane domains and to provide a stop codon following the processing site between the gp120 and gp41 domains of the gp160 protein. This mutagenesis was accomplished by subcloning the fragment which encodes the env gene from clone pSV7c/env (ATCC Accession No. 67593) by excising with HindIII and XhoI (SF2 clone positions 5582 and 8460) and inserting the 2.8 kb fragment into M13mp19 previously digested with HindIII and SalI. A 37 bp oligonucleotide of the following sequence was used to alter the sequence at the gp120/gp41 processing site at position 7306 to encode 2 stop codons and two restriction endonuclease sites.

5'-GAACATAGCTGTCGACAAGCTTCATCATCTTTTTTCT-3'

The sequence of the wild-type gene and the mutant are shown below:

Wild-type sequence:

```
                                            Processing Site
Position:     7288    7294    7300                            7318
Amino acid:   Val Gln Arg Glu Lys Arg Ala Val Gly Ile Val
       DNA:   GTG CAG AGA GAA AAA AGA GCA GTG GGA ATA GTA
```

gp120 mutant:

```
Position:     7288    7294    7300    7306               7317
Amino Acid:   Val Gln Arg Glu Lys Arg OP  OP
       DNA:   GTG CAG AGA GAA AAA AGA TGA TGA AGC TTG TCG AC
Restriction site:                         HindIII SalI
```

Following mutagenesis, the gene was engineered for optimal secretion into the medium. A 268 bp XbaI-NdeI fragment containing the heterologous 5' untranslated sequences and signal sequences from human tPA fused to the 5' end of env was excised from the M13 clone M13tpaS.NheIenv described below. This fragment was ligated with a 1363 bp NdeI-SalI fragment encoding the remainder of the gp120 coding region which was isolated from the gp120 mutant (positions 5954 and 7317) described above, and both fragments were inserted into the vector pSV7d (described below) previously digested with XbaI and SalI.

Expression vector pSV7d can be generated by digesting pSV7c/env with BglII and XbaI, and then ligating the digested plasmid with the following linker:

```
      BglII EcoRI    SmaI  XbaI   BamHI SalI
5'-GATCTCGAATTCCCCGGGTCTAGAGGATCCGTCGAC
        ABCTTAAGGGGCCCAGATCTCCTAGGCACGTGGATC-5'
```

M13tpaS.NheIenv was constructed as follows. The 5′ end of the env coding sequence was modified to accept a heterologous signal sequence known to direct efficient secretion of both the homologous gene (human tissue plasminogen activator) and deletion variants of this gene. van Zonnefeld et al., (1986) Proc. Natl. Acad. Sci. USA 83:4670.

A portion of the HIV-1 SF2 gene in a lambda phage (ATCC Accession No. 40143) was excised with SacI and StuI (positions 5555 and 6395) and was subcloned into the vector M13mp19 [Yanisch-Perron et al., (1985) Gene 33:103-109] between SacI and SmaI. Oligonucleotide-directed mutagenesis [Zoller et al., (1983) Meth. Enzymol. 100:468-500] was used to create an NheI site at the junction of the natural signal peptide and the mature envelope polypeptide using the following oligonucleotide:

5′-GATGCTCTGTTCAGCTAGCGAAAAATTGTGG-3′

This mutagenesis changes cytosine-5867 to guanine and adenine-5868 to cytosine, thereby creating an NheI site and altering the codon for threonine-30 to code for serine.

In parallel, the tPA gene was likewise mutagenized in M13 to place an NheI site near the carboxyl end of the tPA signal peptide. The following sequences show the 5′ UT sequences and signal for wild-type tPA leader and for the NheI variant.

Wild-type sequence of the tPA signal:

5′ untranslated sequences

```
AGAGCTGACATCCTACAGGAGTCCAGGGCTGGAGAGAAAACCTCTGCGAGGAAAGGGAAGGA

GCAAGCCGTGAATTTAAGGGACGCTGTGAAGCAATCATGGATGCAATGAAGAGAGGGCTC
                                   MetAspAlaMetLysArgGlyLeu

TGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTTTCGCCCAGCCAGGAAATCCATGCC
CysCysValLeuLeuLeuCysGlyAlaValPheValSerProSerGlnGluIleHisAla

                      mature tPA
CGATTCAGAAGAGGAGCCAGA TCTTACCAAGTG
ArgPheArgArgGlyAlaArg SerTyrGlnVal
```

The NheI variant of the tPA signal:

5′ untranslated sequences

```
AGAGCTGAGATCCTACAGGAGTCCAGGGCTGGAGAGAAAACCTCTGCGAGGAAAGGGAAGGA

GCAAGCCGTGAATTTAAGGGACGCTGTGAAGCAATCATGGATGCAATGAAGAGAGGGCTC
                                   MetAspAlaMetLysArgGlyLeu

TGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTTTCGCCCAGCGCTAGC
CysCysValLeuLeuLeuCysGlyAlaValPheValSerProSerAlaSer
```

Following mutagenesis and sequence verification, a 174 bp fragment containing 99 bp of 5′ untranslated sequence and the signal sequence from tPA was excised from the tPA-containing M13 clone using SalI and NheI and fused to the 559 bp fragment containing the 5′ end of the env gene which was excised from the env-containing M13 clone with NheI and HindIII (contributed by the M13 polylinker), and these fragments were subcloned into M13mp18 between SalI and HindIII to give plasmid M13tpaS.NheIenv. The DNA and amino acid sequence of the tPA signal fused to the 5′ end of env gene is:

```
 ...tPA signal...         5869 (amino acid 31 of env)
Phe Val Ser Pro Ser Ala Ser Glu Lys Leu Trp Val Thr Val
TTC GTT TCG CCC AGC GCT AGC GAA AAA TTG TGG GTC ACA GTT
                    NheI
```

Example II

This example describes the expression of HIV-1 env analogs in mammalian cells.

DNA encoding the complete env gene with the substituted tPA signal sequence was excised from the plasmid pSV7dARV120tpa using the restriction endonuclease SalI and inserted into the unique SalI site of the mammalian cell expression vector pCMV6a, and the resulting plasmid DNA was screened to verify the correct orientation of the gene with respect to the promoter and polyadenylation signals (see Example I). The resulting plasmid, pCMV6ARV120tpa, and the plasmid pSV7dARV120tpa were used to transfect COS-7 cells to test expression and secretion of gp120 into the medium. pCMV6ARV120tpa was at least 50-fold more efficient in expressing gp120 compared with the pSV7dARV120tpa expression plasmid in these cells.

Permanent cell lines were isolated as follows: human kidney 293 cells were plated at a density of 50-70% confluency in DME supplemented with glutamine (292 mg/L), sodium pyruvate (110 mg/L), glucose (4.5 g/L), penicillin (1000 U/L), streptomycin (1000 U/L), 3.7 g/L sodium bicarbonate, and fetal calf serum (10% v/v). Cells were exposed to a calcium phosphate coprecipitate following standard techniques with 10 ug each of the HIV env expression plasmid pCMV6ARV120tpa (wild type or deletion mutant) and a plasmid encoding the selectable marker neomycin-resistance, pSV2neo (Ref), for six hours at 37°C in a 10% CO_2 atmosphere. Cells were washed and exposed to a 3 to 4 minute shock of 15% DMSO or glycerol in HEPES-buffered saline, and growth medium (described above) was replaced for 48 hours. Trypsinized cells were replated at a lower

density in 400 ug/ml G418 (Sigma) in DME supplemented as above. Colonies grew to the 100 cell per focus stage in one week to ten days, and these colonies were transferred individually to 96 well plates. Clones were screened for gp120 production by testing the conditioned cell medium using an ELISA described below. Positive clones were scaled up to T75 flasks, aliquots of cells frozen, and cell supernatants were collected for further characterization, e.g. CD4 binding.

Alternatively, CHO dhfr- cells plated at a density of 50-70% confluence were cotransfected by calcium phosphate coprecipitation using 10 ug each of the plasmids pCMV6ARV120tpa (or analogous deletion mutant expression vector) and the selectable marker dhfr encoded in the plasmid pAd-dhfr. Following exposure to the coprecipitate and shock solutions as described above, cells were incubated for 48 hours in Ham's F12 supplemented with glutamine (292 mg/L), sodium pyruvate (110 mg/L), sodium bicarbonate (3.7 g/L), glucose (4.5 g/L), penicillin (1000 U/L), streptomycin (1000 U/L), proline (150 mg/L), and fetal calf serum (10%). Forty-eight hours after transfection, cells were plated at a density of approximately one tenth in DME supplemented as described for F12 above, except that the fetal calf serum was replaced with dialyzed fetal calf serum (10%). Colonies were transferred individually to 96 wells after about two weeks, then screened using the ELISA assay for gp120 secreted into the medium. Positive clones were scaled up as described above.

For detection of gp120 or gp120 hypervariable region deletion mutants in the supernatants of COS, CHO, or 293 cells, conditioned medium was assayed by ELISA specific for gp120 sequences. Pooled HIV-positive human serum inactivated by treatment with psoralen was affinity purified on Staphylococcus Protein A Sepharose by standard

techniques. This serum was coated on Immunlon 1 96 well ELISA plates at a concentration of 5 ug/ml in PBS and plates were incubated 12 hours to two months at 4°C. Following incubation, plates were washed as described for the titration ELISA (Example IV), and samples and standards (including purified recombinant HIV-1 gp120env from yeast) were applied to the plate in two-fold dilution series using the dilution buffer described for the titration ELISA. The range of the assay is 100 ng/ml to 200 pg/ml. Samples were incubated for 12 hours at 4°C. Samples were aspirated and plates were washed as above. Samples were then incubated with an appropriate dilution of rabbit serum from rabbits immunized with recombinant SF2 gp120env analog (usually 1:100 dilution of Protein A Sepharose affinity-purified serum in dilution solution) for 1 hour at 37°C, followed by washing. Color development was with ABTS, as described for the titration ELISA. Plates were read as described, and the amount of gp120 in each sample was determined by using a standard curve derived from the standard on the same plate. The assay was verified by showing that HIV-infected HUT 78 cells (infected cell lysate) gave a positive signal, while uninfected cell lysates were negative.

Example III

This example describes the recombinant production of muteins in yeast hosts according to the present invention.

The starting plasmid used for the construction of yeast expression vectors was plasmid pJS150. This plasmid is similar to plasmid pBS24.1/SOD-SF2env4-5 (U.S. serial no. 138,894, filed 24 December 1987, supra), and has had the yeast promoter and HIV coding sequences located between the unique BamHI and SalI sites replaced with a yeast ADH2/GAPDH promoter and a portion of an Zairan HIV-1 isolate envelope coding sequence. In addition, the NheI restric-

tion site located in the plasmid vector portion was destroyed by cutting with NheI, nuclease S1 treatment, followed by ligation.

Plasmid pJS150 was digested with restriction enzyme NcoI, which cuts just after the translation initiation ATG codon downstream from the ADH2/GAPDH promoter (as well as at other sites in the vector). The fragments were ligated to an NcoI/NheI adaptor having the sequence:

```
5'-CATGGCTAGCCCC
       CGATCGGGG-5'
```

After ligation, the DNA sequences were digested with BamHI and NheI to generate a 1.2 kb BamHI-NheI fragment containing the ADH2/GAPDH promoter and an NheI sticky end immediately downstream. This DNA fragment was isolated by gel electrophoresis and is referred to as Fragment A.

A second digest was performed by cutting plasmid pJS150 with BamHI and SalI to generate a 13 kb linear DNA vector. This DNA fragment was also purified by gel electrophoresis, and is referred to as Fragment B. A third DNA fragment containing a coding sequence for gp120 D3 mutein was isolated from pCMV6a120D3 (Table 3 of Example I) by digestion with NheI and SalI. The approximate 1.2 kb coding fragment was then gel isolated, and is referred to as Fragment C.

The three fragments (A, B and C) were then ligated together, and the resulting ligation mix was used to transform E. coli strain HB101 to ampicillin resistance. A plasmid containing all three fragments in the proper orientation is shown in Figure 5. This D3 yeast expression vector was designated pHL15. Expression vectors for additional muteins were also constructed by cloning the mutein-encoding sequence of the pCMV6a-based vectors (Example I, Table 3) into NheI/SalI-digested pHL15, thereby

replacing the D3 coding sequence. The deletions and corresponding vectors that were made are shown in Table 4.

Table 4

Deletion	Vector
D1	pHL24
D2	pHL25
D3	pHL15
D4	pHL26
D5	pHL27
D1 + D2	pHL22
D3 + D4 + D5	pHL21
D1 through D5	pHL20

The yeast expression vectors described above were used to transform Saccharomyces cerivisiae strain JSC308 (ATCC accession No. 20879, deposited 5 May 1988) to uarcil prototrophy. Uracil prototrophs were then streaked onto leucine selective plates to isolate leucine prototrophs (as a result of plamsmid amplification in vivo). Expression of the deletion muteins was achieved by growing a seed culture of the leucine prototrophs in leucine selective medium and then diluting it into approximately 10 liters of a rich medium containing yeast extract, peptone, and glucose. Either 2% or 4% glucose was used as the carbon source in the media, whichever appeared optimal.

Muteins were purified as follows. Frozen yeast cells were thawed and suspended in 1 volume of lysis buffer, 0.001 M PMSF, 0.001 M EDTA, 0.15 M NaCl, 0.05 M Tris-HCl pH 8.0), and 1 volume of acid-washed glass beads added. Cells were broken in a noncontinuous system using a 300 ml glass unit of Dyno-mill at 3000 rpm for 10 min. the jacket was kept cool by a -20°C ethylene glycol solution.

Glass beads are decanted by letting the mixture set for 3 min on ice. The cell extract was recovered and centrifuged at 18,000 rpm (39,200 x g) for 35 min. The supernatant was discarded and the precipitate (pellet 1) further treated as indicated below.

Pellet 1 was resuspended in 4 volumes of Tris-HCl buffer (0.01 M Tris-HCl, pH 8.0, 0.01 M NaCl, 0.001 M PMSF, 1 ug/ml pepstatin, 0.001 M EDTA, 0.1% SDS) and extracted for 1 hr at $4^{o}C$ with agitation. The solution was centrifuged at 6,300 x g for 15 min. The insoluble fraction (pellet 2) was resuspended in 4 volumes (360 ml) of PBS (per liter: 0.2 g KCl, 0.2 g KH_2PO_4, 8.0 g NaCl, 2.9 g $Na_2HPO_4.12H_2O$), 0.1% SDS, 0.001 M EDTA, 0.001 M PMSF, 1 ug/ml pepstatin, and centrifuged at 6,300 x g for 15 min. This pellet (pellet 3), was suspended in 4 volumes of PBS, 0.2% SDS, 0.001 M EDTA, 0.001 M PMSF, 1 ug/ml pepstatin and extracted for 12 hr at $4^{o}C$ with agitation on a tube rocker. The solution was then centrifuged at 6,300 x g for 15 min. The soluble fraction was recovered for further purification as indicated below. (The pellet can be reextracted by resuspending it in 4 volumes of 2.3% SDS, 5% beta-mercaptoethanol, and boiling for 5 min. After boiling, the solution is centrifuged at 6,300 x g for 15 min. The soluble fraction is recovered for further purification.)

The soluble fraction was concentrated by precipitation with 30% ammonium sulfate at $4^{o}C$. The pellet (pellet 4) was then resuspended in 2.3% SDS, 5% beta-mercaptoethanol, and chromatographed on an ACA 34 or ACA 54 (LKB Products) gel filtration column (depending on the size of the mutein). The column was equilibrated with PBS, 0.1% SDS, at room temperature. Chromatography was developed in the same solution with a flow rate of 0.3 ml/min. If needed, pooled fractions were concentrated by vacuum dialysis on Spectrapor #2 (MW cutoff 12-14K).

Example IV

This example describes an immunoassay for anti-HIV antibodies employing HIV-1 env analogs.

Immulon-1 96 well immunoassay plates are coated with gp120 antigen by dispensing 100 ul per well of a 2 ug per ml purified antigen produced in yeast (Env SF2 wild type, env HTLV wild type, env Zr6 wild type, env SF2-D3, env SF2-D1-D2, env SF2-D3-D4-D5, env SF2-D1-D2-D3-D4-D5 in 50 mM borate pH 9.2 at $4^{O}C$ for at least 12 hours and less than 60 days. The coating is aspirated from the plate, and the plate is washed six times by dunking in a solution of 0.137 M NaCl (0.8%), 0.05% Triton-X 100. The plate is patted dry, and 100 ul per well of 100 mM sodium phosphate, 0.1% casein, 1 mM EDTA, 1% Triton-X 100, 0.5 M NaCl, 0.01% thimerosol pH 7.5 (dilution solution) is added.

Sera to be tested are prepared for analysis by diluting 5 ul test serum (from HIV-positive humans and normal humans, or from immunized animals) in 500 ul of the dilution solution above (1/100 dilution v/v). The solution in the top wells is aspirated off and 150 ul of diluted test serum is added. Using a multichannel pipettor set at 50 ul, dilutions down each column are carried out, taking 50 ul each time (1/3 dilution v/v). The plates are then incubated 1 hour at $37^{O}C$ with the plate wrapped in plastic wrap. The samples are then aspirated off and washed 6 times as above. Then, 100 ul per well of goat anti-human IgG conjugated to horseradish peroxidase (Tago 2733 Lot 330102) diluted 1/2000 in dilution solution is added. The plates are then incubated 30 minutes at $37^{O}C$ covered in plastic wrap. The solution is again aspirated off and washed 6 times as above. 100 ul per well of color developing solution is added [100 ul ABTS stock (15 mg/ml 2,2'-A zino-di-(3 ethylbenzthiazolene sulfonic acid), Sigma A-1888, in water, stored in the dark at $4^{O}C$) plus 3.3 ul 30% hydrogen peroxide in 10 ml citrate buffer (10.5 g citric

acid per liter water, pH to 4.0 with 6M NaOH)]. The ABTS solution is made no more than 10 minutes prior to use, and the solution should be made with citrate buffer at room temperature. Plates in plastic wrap are incubated at 37^{O} for 30 minutes in the dark. The reaction was stopped with 50 ul per well of 10% sodium dodecyl sulfate.

Plates were read at 415 nm with a reference wavelength at 600 nm. Titers are determined as follows from the raw data: absorbance (linear axis) is plotted vs. dilution factor (log axis) on semilog paper or computer program.

Included in the test sera are standard reference sera as positive controls. For titration of human sera, HIV-positive serum 20058 from the Interstate panel was used; for goat sera, a goat serum developed by immunization with envSF2 wild-type, reference 02GT097.2 was used; and for titration of guinea pig sera, a standard reference guinea pig serum was used, likewise obtained by immunization with envSF2 wild-type, reference +Gp sera 1935/77. From the values obtained on the ELISA reader, an absorbance value was chosen that is half-maximal (OD_{50}) (between 0.5 and 0.7) in the linear portion of the standard curve (positive control), for each plate. The average titer for the standards on the plate were determined. The average titer was divided by the "Reference Titer" for that species and antigen plate, and the resulting number is the "correction factor". The test sera titers were divided by the correction factor to yield the adjusted, normalized titers.

A panel of selected human sera was tested to determine their titers on all of the recombinant antigens, including the deletion muteins env SF2 D1-2, env SF2-D-3, env SF2-D1-2-3-4-5, and env SF2-D3-4-5. Results are shown in Figure 6 (North American serum panel) and Figure 7 (African serum panel). The graphs show that for these serum samples, the recombinant antigen env SF2-D1-2 is as

efficient or more efficient in detecting sera as is env SF2 wild-type. Recombinant antigens env SF2-D3, env SF2-D3-4-5 and env SF2-D1-2-3-4-5 are as efficient as env HTLVIII wild-type or env Zr6 wild-type.

Example V

This example describes the immunization of mammals with recombinant hypervariable region muteins and the detection of immune responses in these animals in response to these injections. Recombinant env antigens purified from yeast were used to generate anti-HIV antibodies in experimental animals of very high titer. In both goats and guinea pigs, the titers of the resulting sera were at a similar level, whether the immunogen was env SF2 wild-type or an env SF2 hypervariable mutein.

Immunization of Guinea Pigs

In order to test if guinea pigs imunized with env SF2 muteins derived from yeast were capable of generating a strong immune response, these animals were immunized with several muteins and control antigens.

For each antigen, six Hartley guinea pigs were immunized in the footpad with 50 ug each antigen mixed with 50 ug adjuvant (see below) at three week intervals with a total of seven injections. Blood samples were taken at the time of each injection (prebleed at injection 1), and the serum was monitored by titration in the ELISA described above in Example IV for the production of antibodies directed against the immunizing antigen and against heterologous env antigens.

Antigens:

Env SF2-D3
Env SF2-D-1-2-3-4-5
Env SF2-D3-4-5
Env HTLVIII wild-type

Adjuvant:

Muramyl-tripeptide-phosphatidyl ethanolamine in squalene-Tween.

Vaccine:

Mix adjuvant with correct volume of 10X Squalene-Tween (carrier) to make a final concentration of 1X (4% squalene, 0.008% Tween-20), antigen, and PBS to make the correct volume and dose (50 ug each of antigen and adjuvant per animal in 100 ul injection volume per animal. Warm the mixture for 5 min at 45°C. Pass the warm mixture in and out of a 23 ga. needle six times, taking care to avoid introducing air into the mixture. Inject into the animals immediately. If the injection procedure takes more than 5 min, remix the emulsion by shaking by hand every few minutes.

Control:

Control animals receive the adjuvant in carrier.

Results:

The results of the ELISA are shown in Figure 8. As can be seen, the muteins of the present invention are as effective as wild-type envelope in generating high antibody titers. A standard virus neutralization assay was also conducted the guinea pig sera. The results are shown in Table 5. The data show that deletion mutants of the present invention can generate significant levels of neutralizing antibodies.

Table 5

Virus Neutralization by Guinea Pig Sera

Antigen	Animal Number	ELISA Titer/ env SF2	Neutralization Titer HIV-SF2
Env SF2 D3 (COOH half)	2477	280,000	50
	2476	221,000	500
	2479	274,000	250
	2480	190,000	100
	2481	115,000	<20
Env SF2 D3	2471	45,000	>500
	2473	57,000	<20
	2474	116,000	<20
	2475	26,000	<20
	2476	16,000	<500
Env SF2 D(3-5) (full length)	2489	342,000	500
	2490	88,000	30
	2491	131,000	45
	2492	83,000	25
	2494	253,000	30
Env SF2 D(1-5)	2483	131,000	<20
	2484	71,000	<20
	2485	60,000	<20
	2486	54,000	<20
	2487	100,000	<20
	2488	133,000	<20
Env HTLVIII	2523	23,000	<20
	2524	37,000	<20
	2525	52,000	<20
	2526	23,000	<20
	2527	70,000	<20
	2528	14,000	<20

Immunization of Goats

In order to test if goats immunized with env SF2 muteins derived from yeast were capable of generating a strong immune response, these animals were immunized with several muteins and control antigens.

For each antigen, two goats were immunized intramuscularly with 1 mg each primary injection (complete Freund's adjuvant) and 0.5 mg each booster injection (incomplete Freund's adjuvant) at three week intervals with a total of six injections. Blood samples were taken at the time of each injection (prebleed at injection 1), and the sera were monitored by titration in the ELISA assay described above in Example IV for the production of antibodies directed against the immunizing antigen and against heterologous env antigens.

Antigens:	Env SF2-D3	Lot 3064/1a
	Env SF2-D1-a2-3-4-5	Lot 3064/5a
	Env HTLVIII wild-type	Lot 3064/15a

Adjuvant: Complete Freund's and incomplete Freund's.

Vaccine: Mix 0.5 ml antigen (1 mg) with 0.5 ml complete Freund's. Emulsify by standard procedures and inject. For boosters, mix 0.5 ml antigen (0.5 mg) with 0.5 ml incomplete Freund's. Emulsify and inject.

Control: Control animals receive the adjuvant in carrier.

Results: The results of the ELISA are shown in Figure 9. As can be seen, the muteins of the present invention are at least as effective as wild-type polypeptides in antibody titer levels generated.

Deposit of Biological Materials

Vectors pCMV6ARV120tpa and pHL15, both in E. coli HB101, were deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, USA, on 13 September 1988, and will be maintained under the provisions of the Budapest Treaty on the Deposit of Microorganisms. The accession number for the pCMV6ARV120tpa deposit is 67792, and the accession number for the pHL15 deposit is 67793.

These deposits are provided for the convenience of those skilled in the art, and are neither an admission that such deposits are required to practice the present invention, nor that equivalent embodiments are beyond the skill of the art in view of the present disclosure. The availability of these deposits is not a grant of any license (e.g., to make, use or sell the deposited materials) under this or any other patent. The nucleic acid sequences of the deposited materials are incorporated in the present disclosure by reference and are controlling if in conflict with any sequence described herein.

Although the foregoing invention has been described in some detail by way of illustration and example, it will be obvious that changes and modifications may be practiced within the scope of the apended claims.

CLAIMS

1. An improved polypeptide analog of human immunodeficiency virus type 1 (HIV-1) gp120env or gp160env, the improvement comprising the deletion of at least one epitope in a hypervariable domain, while retaining the domains conserved among HIV-1 isolates.

2. A polypeptide comprising epitopes bound by antibodies to HIV-1 gp120env or gp160env and an amino acid sequence according to the formula:

C1-V1-V2-C2-V3-C3-V4-C4-V5-C5

wherein:

C1 is an amino acid sequence substantially homologous to Ser29 through Cys130 of HIV-1 SF2;

C2 is an amino acid sequence substantially homologous to Cys199 through Leu291 of HIV-1 SF2;

C3 is an amino acid sequence substantially homologous to Ser366 through Cys387 of HIV-1 SF2;

C4 is an amino acid sequence substantially homologous to Cys415 through Gly456 of HIV-1 SF2;

C5 comprises an amino acid sequence substantially homologous to Phe466 through Arg509 or Leu855 of HIV-1 SF2;

V1 is an amino acid sequence of 0 to a maximum of about 30 residues;

V2 is an amino acid sequence of 0 to a maximum of about 50 residues;

V3 is an amino acid sequence of 0 to a maximum of about 90 residues;

V4 is an amino acid sequence of 0 to a maximum of about 30 residues; and

V5 is an amino acid sequence of 0 to a maximum of about 10 residues;

with the proviso that at least one of the V domains selected from the group consisting of V1, V2, V3, V4 and V5 contains no more than about one-third of the said maximum number of residues for the V domain.

3. The polypeptide of claim 2 wherein:

V1, if present, comprises an HIV-1 sequence spanning from the C-termini of C1 to a Cys corresponding to the conserved Cys155 of HIV-1 SF2;

V2, if present, comprises an HIV-1 sequence spanning from said C-termini Cys of V1 to the N-termini of C2;

V3, if present, comprises an HIV-1 sequence spanning from the C-termini of C2 to the N-termini of C3;

V4, if present, comprises an HIV-1 sequence spanning from the C-termini of C3 to the N-termini of C4; and

V5, if present, comprises an HIV-1 sequence spanning from the C-termini of C4 to the N-termini of C5;

with the proviso that for said selected V domain having no more than one-third the maximum residue, if said HIV-1 sequence is present, said HIV-1 sequence is missing an epitope.

4. The polypeptide of claim 2 wherein said selected V domain is V1.

5. The polypeptide of claim 2 wherein said selected V domain is V2.

6. The polypeptide of claim 2 wherein said selected V domain is V3.

7. The polypeptide of claim 2 wherein said selected V domain is V4.

8. The polypeptide of claim 2 wherein said selected V domain is V5.

9. The polypeptide of claim 2 wherein said selected V domains are V1 and V2.

10. The polypeptide of claim 2 wherein said selected V domains are V1, V2 and V3.

11. The polypeptide of claim 2 wherein said selected V domains are V1, V2 and V5.

12. The polypeptide of claim 2 wherein said selected V domains are V3, V4 and V5.

13. The polypeptide of claim 2 wherein said selected V domains are V1, V2, V3, V4 and V5.

14. A polypeptide analog of human immunodeficiency virus type 1 (HIV-1) gp120env or gp160env comprising (a) about 300 to about 850 amino acid residues in length; (b) constant domains, in the N-termini to the C-termini direction, Ser29-Cys130 of HIV-1 SF2 (C1), Cys199-Leu291 of HIV-1 SF2 (C2), Ser366-Cys387 (C3), Cys415-Gly456 (C4) of HIV-1 SF2, and Phe466-Arg509 or Phe466-Leu855 of HIV-1 SF2 (C5), or domains substantially homologous to said C1, C2, C3, C4 or C5; and (c) the intervening domains, if any, located between said constant domains comprising sequences found between said constant domains in native HIV-1 gp120env, with the proviso that at least one of said intervening domains between said constant domains is either missing or missing an epitope.

15. The polypeptide of claim 14 wherein said missing epitope is a neutralizing epitope.

16. The polypeptide of claim 14 wherein said intervening domains are HIV-1 SF2 sequences.

17. The polypeptide of claim 16 comprising an HIV-1 SF2 gp120env deletion mutein wherein C5 is Phe466-Arg509 and at least one deletion is selected from the group consisting of:

(a) Thr131-Asn154;
(b) Ser156-Ser198;
(c) Thr300-His332;
(d) Asn388-Pro414; and
(e) Thr457-Thr463;

or a polypeptide substantially homologous thereto.

18. The polypeptide of claim 17 wherein said deletion comprises Thr131-Asn154.

19. The polypeptide of claim 17 wherein said deletion comprises Ser156-His198.

20. The polypeptide of claim 17 wherein said deletion comprises Thr300-His332.

21. The polypeptide of claim 17 wherein said deletion comprises Asn388-Pro414.

22. The polypeptide of claim 17 wherein said deletion comprises Thr457-Thr463.

23. The polypeptide of claim 17 wherein said deletions comprise Thr131-Asn154 and Ser156-His198.

24. The polypeptide of claim 17 wherein said deletions comprise Thr131-Asn154, Ser156-His198 and Thr457-Thr463.

25. The polypeptide of claim 17 wherein said deletions comprise Thr300-His332, Asn388-Pro414 and Thr457-Thr463.

26. The polypeptide of claim 17 wherein said deletions comprise Thr131-Asn154, Ser156-His198, Thr300-His332, Asn388-Pro414 and Thr457-Thr463.

27. An immunoassay for the detection of antibodies to human immunodeficiency virus type 1 (HIV-1) comprising:

(a) providing a liquid sample to be tested for the presence of anti-HIV-1 antibodies;

(b) contacting said sample with a polypeptide according to claim 1 under conditions whereby any anti-HIV-1 antibodies present in said sample may bind to an epitope to provide sample-contacted polypeptide; and

(c) detecting any antibody bound to said sample-contacted polypeptide.

28. A method of selectively raising antibodies in a mammal to epitopes in the constant domains of human immunodeficiency virus type 1 (HIV-1) gp120env or gp160env comprising administering to said mammal a polypeptide of claim 1, whereby antibodies to said polypeptide are produced in response to said administration.

29. A composition comprising a polypeptide according to claim 1 and a pharmaceutically acceptable carrier.

30. The composition of claim 29 further comprising an adjuvant.

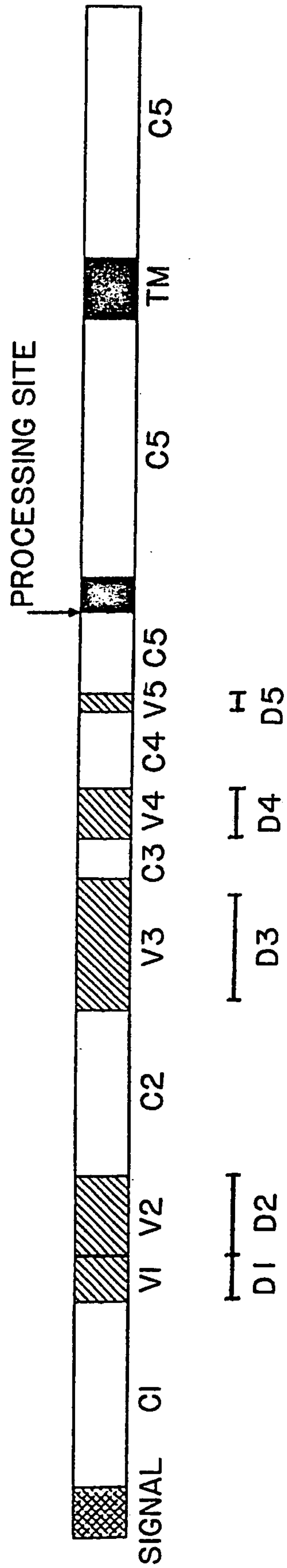
31. A DNA sequence encoding a polypeptide according to claim 1.

32. A cellular host comprising the DNA sequence of claim 31 under the control of transcriptional and translational control sequences whereby the polypeptide encoded by said DNA sequence is expressed by said cellular host.

33. The cellular host of claim 32 wherein said cellular host is a mammalian cell.

34. The cellular host of claim 32 wherein said cellular host is a yeast cell.

35. A method of producing a recombinant analog of human immunodeficiency virus type 1 (HIV-1) envelope protein comprising growing culture of cellular hosts according to claim 32 under conditions whereby said polypeptide encoded by said DNA sequence is expressed, and recovering said polypeptide from said culture.



HIV—SF2env
2565bp

FIG. 1

```
HXB2  MetArgValLysGluLys.........TyrGlnHisLeuTrpArgTrpGlyTrpArgTrp   17
BRU   ---------------------------------------------------Lys---   17
MN    ------------GlyIleArgArgAsn---------...---...----------......   16
SC    ------------GlySerGlyArgAsn----------------------............   16
SF2   ---Lys------GlyThrArgArgAsn----------------------............   16
NY5   ------Ala---GlyThrArgLysAsn----------------------............   16
CDC4  ------Ala---GlyIleArgLysAsnCys-------------------............   16
WMJ2  ------------GlyIleMetArgAsnCys------------Ile---............   16
RF    ---------Met---MetArgLysAsnCys------------Lys---............   16
MAL   ---------Arg---IleGlnArgAsn------AsnTrp---------............   16
ELI   ------AlaArgGlyIleGluArgAsnCys---AsnTrp---Lys---............   16
Z6    ------AlaArg---IleGluArgAsnCysProAsn------Lys---............   16
Z3    ---------------IleGlnArgAsn---------------Lys---............   16
Z321  ---Lys------GlyIleGlnGlyAsnTrp---AsnTrp---Lys---............   16
JY1   ---------MetGlyIleArgMetAsn---------------Lys---............   16
```

FIG. 2-1A

```
                          signal peptide \/ gp120
                                        *
HXB2   GlyThrMetLeuLeuGlyMetLeuMetIleCysSerAlaThrGluLysLeuTrpValThr   37
BRU    ------------------Ile---------------------------------------   37
MN     ------------------Leu---------------------------------------   36
SC     ------------------Ile---------------Ala---Gln---------------   36
SF2    ------Leu---------------------------------------------------   36
NY5    ------------------------------------------------------------   36
CDC4   ------------------------------------AlaAlaAsn---------------   36
WMJ2   ------------Phe------Trp------------Val---Gln---------------   36
RF     ------------------------------------Ala---Asp---------------   36
MAL    ---Met---------------------Thr------IleAla---Asp---------------   36
ELI    ---Ile------------Ile------Thr------AlaAspAsn---------------   36
Z6     ---Ile------------Ile---------------AlaAspAsn---------------   36
Z3     SerLeuIleIle---------Ile---------Lys---Ile---------Ser---------   36
Z321   ------LeuIle------LeuValIle---------Ala---Asn---------------   36
JY1    ---Ile------------Ile------Thr---ValAla---Asp---------------   36
```

CL

FIG. 2-IB

```
                                                           *
HXB2   ValTyrTyrGlyValProValTrpLysGluAlaThrThrThrLeuPheCysAlaSerAsp   57
BRU    ------------------------------------------------------------   57
MN     ------------------------------------------------------------   56
SC     ------------------------------------------------------------   56
SF2    ------------------------------------------------------------   56
NY5    ------------------------------------------------------------   56
CDC4   ------------------------------------------------------------   56
WMJ2   ------------------------------------------------------------   56
RF     ---------------------------------------------------------Glu   56
MAL    ------------------------------------------------------------   56
ELI    ------------------------------------------------------------   56
Z6     ------------------------------------------------------------   56
Z3     ---------------------------Asp---Glu------------------------   56
Z321   ---------------------------Asp---Glu------------------------   56
JY1    ------------------------------------------------------------   56
```

FIG. 2-2A

																	*				
HXB2	Ala	Lys	Ala	Tyr	Asp	Thr	Glu	Val	His	Asn	Val	Trp	Ala	Thr	His	Ala	Cys	Val	Pro	Thr	77
BRU	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	77
MN	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Gln	—	—	—	—	—	76
SC	—	—	—	—	—	—	—	—	—	—	Ile	—	—	—	—	—	—	—	—	—	76
SF2	—	Arg	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	76
NY5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	76
CDC4	—	—	—	—	—	—	—	Ala	—	—	—	—	—	—	—	—	—	—	—	—	76
WMJ2	—	—	—	—	Ser	—	—	Ala	—	—	—	—	—	—	—	—	—	—	—	—	76
RF	—	—	—	—	Lys	—	—	—	—	—	—	—	—	Lys	—	—	—	—	—	—	76
MAL	—	—	Ser	—	Glu	—	—	—	—	—	Ile	—	—	—	—	—	—	—	—	—	76
ELI	—	—	Ser	—	Glu	—	—	Ala	—	—	Ile	—	—	—	—	—	—	—	—	—	76
Z6	—	—	Ser	—	Lys	—	—	Ala	—	—	Ile	—	—	—	—	—	—	—	—	—	76
Z3	—	—	—	—	Glu	Lys	—	Ser	—	—	—	—	—	—	—	—	—	—	—	Ser	76
Z321	—	—	—	—	—	—	—	Lys	—	—	—	—	—	—	—	—	—	—	—	—	76
JY1	—	—	Ser	—	Glu	Pro	—	Ala	—	—	Ile	—	—	—	—	—	—	—	—	—	76

FIG. 2-2B

```
                                        [      ]
HXB2  AspProAsnProGlnGluValValLeuValAsnValThrGluAsnPheAspMetTrpLys  97
BRU   ------------------------------------------------Asn---------  97
MN    ---------------------Glu------------------------Asn---------  96
SC    ---------------------------Gly------------------Asn---------  96
SF2   ---------------------------Gly------------------Asn---------  96
NY5   ---------------------------Gln------------------Asn---------  96
CDC4  Asn------------------------Glu------------------Asn---------  96
WMJ2  ---------------------Ile---Gly------------------Asn---------  96
RF    ---------------------Leu---Glu------------------Asn---------  96
MAL   ------------------IleGlu---Glu------------Gly---Asn---------  96
ELI   ------------------IleAla---Glu------------------Asn---------  96
Z6    ------------------IleGlu---Glu------------------Asn------Arg  96
Z3    ------Ser---------Leu------Gly------------------Asn---------  96
Z321  ------------------LeuSer---Gly------------Lys---------------  96
JY1   ------------Arg---IleGluMetGlu------------------Asn---------  96
```

FIG. 2-2C

```
HXB2  AsnAspMetValGluGlnMetHisGluAspIleIleSerLeuTrpAspGlnSerLeuLys  117
BRU   ------------------------------------------------------------  117
MN    ---Asn------------------------------------------------------  116
SC    ---Asn------------------------------------------------------  116
SF2   ---Asn---------------Gln------------------------------------  116
NY5   ---AsnThr---------------------------------------------------  116
CDC4  ---Asn------------------------------------------------------  116
WMJ2  ---Asn------------------------------------------------------  116
RF    ---Asn------------------------------------------------------  116
MAL   ---Asn------------------------------------------------------  116
ELI   ---Asn------------------------------------------------------  116
Z6    ---Asn------------Ile---------------------------------------  116
Z3    ---Lys------------------------------------------------------  116
Z321  ---Asn------------------------Val---------------------------  116
JY1   ---Asn---------------------------------------------Asn------  116
```

FIG. 2-3A

```
                                                 CI <--|--> DI
         *                    *           *
HXB2  ProCysValLysLeuThrProLeuCysValSerLeuLysCys|ThrAspLeuLys......   135
BRU   ------------------------------------------|---------Gly------   135
MN    ------------------------------Thr---Asn---|---------Arg------   134
SC    ------------------------------Thr---Asn---|---Asn---Arg------   134
SF2   ------------------------------Thr---Asn---|---------GlyLys---   135
NY5   ------------Ser---------------Thr---Asn---|---------Thr------   134
CDC4  ------------------------------Thr---Asn---|---------AsnThr---   135
WMJ2  ------------------------------Thr---Asn---|Ile---Lys...------   133
RF    ------------------------------Thr---Asn---|------AlaAsnLeu---   135
MAL   ------------------------------Thr---Asn---|---AsnVal...------   133
ELI   ------------------------------Thr---Asn---|Ser---GluLeuArg---   135
Z6    ------------------------------Thr---Asn---|------GluSerAspGlu   136
Z3    ------------------Phe---------Thr---Asn---|Ile---Val---------   134
Z321  ------------------------------Thr---Ser---|HisAsnIleThrIleLys   136
JY1   ------------------------------Thr---Asn---|---AsnAlaGlyGly---   135
```

FIG. 2-3B

```
                  [        ]          [        ]
HXB2  .........AsnAspThr...AsnThrAsnSerSer.....................Ser   144
BRU   ------------Ala---------------------------AsnThrAsnSerSer---   149
MN    ------------Thr----------------Asn---ThrAlaAsnAsnAsn---------   148
SC    ---------------------Ser------AlaThr------AsnThrThrSerSerAsn   148
SF2   ---------...Ala---------------------------AsnTrpLys------...   145
NY5   ------------Ala---TyrAla...---Gly------------------------...   142
CDC4  ------------Asn------...------ThrThrGluLeuSerIleIleValVal...   149
WMJ2  ------------Ile---AspTrpLys---ThrThr---------IleIle------...   144
RF    ------------Gly------......---ValThr---------------SerSer---   144
MAL   ------------Gly---AlaVal...---GlyThrAsnAlaGlySerAsnArgThrAsn   149
ELI   ------Asn---Gly---MetGlyAsn---ValThr---------------------...   145
Z6    TrpMetGly---Val---GlyLys...---ValThr---------------------...   147
Z3    ------------Ser---------...---AsnThr---------------------...   141
Z321  Asp---------Asn---------...ValAspThr---------------------...   144
JY1   ------------Lys---Thr---Gly---AsnThr---------------ThrAsnGln   147
```

FIG. 2-3C

```
[  *  ]  [     ]
```

```
                                                     D1 <-|    |-> D2
                                                        [   *   ]  [       ]
HXB2   GlyArgMetIleMetGluLys...GlyGluIleLysAsnCysSerPheAsnIleSerThr      163
BRU    ---Glu---Met----------------------------------------------------   168
MN     AsnSerGluGlyThrIle---Gly------Met-----------------------Thr---     168
SC     ArgGly...Lys------Gly----------MetThr-------------------Thr---     166
SF2    ......GluGlu...Ile--------------------------------------Thr---     161
NY5    ...SerGluGluArg......-------------Arg----------------ValThr---     158
CDC4   ...TrpGluGlnArgGly------------MetArg--------------------Thr---     167
WMJ2   ..................GlyGly------Val-----------------------Thr---     158
RF     ---GlyThrMet------Asn------------------------------GlnValThr---    163
MAL    AlaGluLeuLys------Ile----------Val----------------------ThrPro     168
ELI    ...ThrGluGlu......---Gly......Met-------------------ValThr---      16Ø
Z6     ......GluAspIleArg...---......Met-----------------------Thr---     161
Z3     ......GluGluAlaThr...---......---Thr----------------LysValPro---   155
Z321   ............GluMet------Glu------------------Tyr---MetThr---       159
JY1    GluGluGlnMet------------------Met-----------------------Thr---     166
```

FIG. 2-4A

HXB2	SerIleArgGlyLysValGlnLysGluTyrAlaPhePheTyrLysLeuAspIleIlePro	183
BRU	--	188
MN	---------Asp---Met---------------LeuLeu---------------ValSer	188
SC	---------Ser---------------------Leu---------------ValVal---	186
SF2	---------Asp---Ile---------Asn---Leu---ArgAsn------ValVal---	181
NY5	Ile------Asn---Ile---------------Leu------Arg---------Val---	178
CDC4	---------Asp---------Arg---------Leu---------------ValGlu---	187
WMJ2	---Arg---Asp------His------------Leu---------------ValVal---	178
RF	---Arg---Asp---Thr------Lys------Leu---------------ValVal---	183
MAL	ValGlySerAsp---Arg---...---------Thr------Asn------LeuValGln	187
ELI	ValLeuLysAsp---Lys---GlnVal------Leu------Arg---------Val---	180
Z6	ValVal---Asp---ThrLysGlnValHis---Leu------Arg---------Val---	181
Z3	GluLeuLysAsp---ThrGluThrValHisThrLeu---------------ValVal---	175
Z321	GluLeu---Asp---GlnArg---Ile---SerLeu------Arg---------Val---	179
JY1	Val---SerAsp---LysLysGlnValHis---Leu------Arg------ValVal---	186

FIG. 2-4B

```
                         [        ]    "peptide T" region
HXB2  Ile..................AspAsnAspThr........................Thr    189
BRU   -------------------------------------------------------------    194
MN    ------------------------------Ser----------------------------    194
SC    ---------------------------......----------------------------    190
SF2   ---------------------------AlaSer---------ThrThrThrAsnTyr---    192
NY5   ------------AspLys---------......----------------------------    184
CDC4  ------------Asp-------------LysAsn------------ThrThrAsnAsn---    198
WMJ2  ---------------LysGly------AsnSer--------------------------Ser    186
RF    ------------GluLysGly...---IleSerProLysAsnAsnThrSerAsnAsn---    199
MAL   ---AspAspSer---------------......-------------------------Ser    194
ELI   ------------------------------Ser------SerThrAsnSer----------    190
Z6    ------------------------------Asn------SerThrAsnSer----------    191
Z3    Leu------------------...---Val---AsnAsnSerSerIleSer-------Ser    186
Z321  ---GlyGly------------...---......------SerSerAsnGlyAspSerSer    190
JY1   ---AspAspAspAsnSerAla...---ThrSer---Asn---------ThrAsnTyr---    201
```

FIG. 2-4C

```
                                      D2 <---> C2
                                              * [       ]            *
HXB2  SerTyrSer.........LeuThrSerCysAsnThrSerValIleThrGlnAlaCysPro   206
BRU   ------Thr---------------------------------------------------   211
MN    ------Arg------------Ile------------------------------------   211
SC    ------Thr------------IleAsn---------------------------------   207
SF2   Asn---Arg------------IleHis------Arg------------------------   209
NY5   ------Thr------------IleAsn---------------------------------   201
CDC4  Lys---Arg------------IleAsn---------------------------------   215
WMJ2  Arg---Arg------------IleAsn---------------------------------   203
RF    ------GlyAsnTyrThr---IleHis------Ser------------------------   219
MAL   ------Arg------------IleAsn---------------------------------   211
ELI   Asn---Arg------------IleAsn------------Ala------------------   207
Z6    Asn---Arg------------IleAsn------------Ala------------------   208
Z3    Thr---Arg------------IleAsn------------Thr------------------   203
Z321  Lys---Arg------------IleAsn------------Ala------------------   207
JY1   Asn---Arg------------IleAsn------------Ala------------------   218
```

FIG. 2-5A

```
                                     *
HXB2  LysValSerPheGluProIleProIleHisTyrCysAlaProAlaGlyPheAlaIleLeu  226
BRU   ------------------------------------------------------------  231
MN    ---Ile------------------------------------------------------  231
SC    ------------------------------------------ArgTrp...---------  226
SF2   ------------------------------------Thr---------------------  229
NY5   ------------------------------------------------------------  221
CDC4  ------------------------------------Thr---Thr---------Leu---  235
WMJ2  ------------------------------------------------------------  223
RF    ------------------------------------Thr---------------------  239
MAL   ------Thr---Asp---------------------------------------------  231
ELI   ------------------------------------------------------------  227
Z6    ------------------------------------------------------------  228
Z3    ------------------------------------------------------------  223
Z321  ------------------------------------------------------------  227
JY1   ------Thr---------------------------------------------------  238
```

FIG. 2-5B

```
          *     [     ]  [     ]        *  [     ]
HXB2  LysCysAsnAsnLysThrPheAsnGlyThrGlyProCysThrAsnValSerThrValGln  246
BRU   ------------------------------------------------------------  251
MN    ---------Asp---Lys---Ser---Lys---Ser---Lys------------------  251
SC    Asn------------Lys------------------------------------------  246
SF2   ---------------------------Lys------------------------------  249
NY5   ---------Asp---Lys------------------------------------------  241
CDC4  ---------Asp---Lys------------------------------------------  255
WMJ2  ---------Asp---Lys------------------------------------------  243
RF    ---------Asp---Lys-----------------------Lys----------------  259
MAL   ---------Asp---Lys---------------GluIle---Lys----------------  251
ELI   ------ArgAsp---Lys------------------------------------------  247
Z6    ------ArgAsp---Arg------------------------------------------  248
Z3    ---------Asp---Lys-----------------------Lys----------------  243
Z321  ------ArgAspGluGlu---Glu---Lys-----------Arg----------------  247
JY1   ------LysAsp---Lys-----------------------LysLys-------------  258
```

FIG. 2-5C

```
       *                                                         [        ]
HXB2   CysThrHisGlyIleArgProValValSerThrGlnLeuLeuLeuAsnGlySerLeuAla   266
BRU    ------------------------------------------------------------   271
MN     ------------------------------------------------------------   271
SC     ------------------------------------His---------------------   266
SF2    ---------------------Ile------------------------------------   269
NY5    ---------------Lys------------------------------------------   261
CDC4   ------------------------------------------------------------   275
WMJ2   ------------------------------------------------------------   263
RF     ------------------------------------------------------------   279
MAL    ---------------Lys------------------------------------------   271
ELI    ------------------------------------------------------------   267
Z6     ------------------------------------------------------------   268
Z3     ---------------------------------------------------------Ser   263
Z321   ------------------------------------------------------------   267
JY1    ------------------------------------------------------------   278
```

FIG. 2-6A

									[			]								
HXB2	Glu	Glu	Glu	Val	Val	Ile	Arg	Ser	Val	Asn	Phe	Thr	Asp	Asn	Ala	Lys	Thr	Ile	Ile	Val	286
BRU	-	-	-	-	-	-	-	-	Ala	-	-	-	-	-	-	-	-	-	-	-	291
MN	-	-	-	-	-	-	-	-	Glu	-	-	-	-	-	-	-	-	-	-	-	291
SC	-	-	-	-	-	Leu	-	-	Glu	-	-	-	-	-	-	-	-	-	-	-	286
SF2	-	-	-	-	-	-	-	-	Asp	-	-	-	Asn	-	-	-	-	-	-	-	289
NY5	-	Gly	-	-	-	-	-	-	Glu	-	-	-	Asn	-	-	-	-	-	-	-	281
CDC4	-	-	-	-	-	-	-	-	Glu	-	-	-	Asn	-	-	-	-	-	-	-	295
WMJ2	-	-	-	Ile	-	-	-	-	Glu	-	-	-	-	-	-	-	-	-	-	-	283
RF	-	-	-	-	-	-	-	-	Glu	-	-	-	-	-	Val	-	-	-	-	-	299
MAL	-	-	-	Ile	Met	-	-	-	Glu	-	Leu	-	-	-	Thr	-	Asn	-	-	-	291
ELI	-	-	-	-	Ile	-	-	-	Glu	-	Leu	-	Asn	-	-	-	Asn	-	-	Ala	287
Z6	-	-	-	Ile	Ile	-	-	-	Glu	-	Leu	-	Asn	-	-	-	Ile	-	-	-	288
Z3	-	-	-	-	Ile	-	-	-	Glu	-	Ile	-	Asn	-	-	-	-	-	-	-	283
Z321	-	Gly	-	-	Arg	-	-	-	Glu	-	-	-	-	-	-	-	Ile	-	-	-	287
JY1	-	-	-	Ile	Ile	-	-	-	Glu	-	Leu	-	Asn	-	Val	-	-	-	-	-	298

FIG. 2-6B

```
           C2 <-|                         |-> D3
                [        ]       [    *   ]      [            ]
HXB2  GlnLeuAsnThrSerValGluIleAsnCysThrArgProAsnAsnAsn...ThrArgLys   305
BRU   ------------Gln--------------------------------------------    310
MN    His---------Glu------Gln--------------------Tyr------Lys---    310
SC    ------LysGluAla----------------------------------------ThrArg  305
SF2   ------------Glu------Ala-----------------------------------    308
NY5   ------------Lys--------------------------------------Lys---    300
CDC4  ------------Val--------------------------------His---------    314
WMJ2  His---------Glu------------------------Tyr--------Val---Arg    302
RF    ------------Ala------Gln-----------------------------------    318
MAL   ------------GluThr---Thr-----------------Gly--------------Arg  310
ELI   His---------Glu------Lys---Thr---Ala------TyrGln----------Gln  306
Z6    ------------Glu------Ala------------------TyrLys----------Gln  307
Z3    ------------GluThr---Lys------------------GlySerAsp---LysLysIle 302
Z321  ------ValLysPro---Asn---Thr---Met---------------------------    306
JY1   His---------Glu---------------------------Asp---LysIle------Gln 318
```

FIG. 2-6C

FIG. 2-7A

```
HXB2   Arg.........IleArgIleGlnArgGlyProGlyArgAlaPheValThrIleGlyLys   322
BRU    Ser---------------------------------------------------------   327
MN     ---------------His---......------------------Tyr---ThrLysAsn   325
SC     Ser------------His---......------------------TyrAlaThr---Asp   320
SF2    Ser------------Tyr---......------------------His---Thr---Arg   323
NY5    Gly------------Ala---......------------ThrLeuTyrAlaArgGlu---   315
CDC4   ---ValThrLeu...............------------ValTrpTyr---Thr---Glu   329
WMJ2   Ser---------LeuSer---......------------------Arg---Arg...Glu   316
RF     Ser------------ThrLys......------------ValIleTyrAlaThr---Gln   333
MAL    Gly------------HisPhe......---------Gln---LeuTyr---Thr---...   324
ELI    ------------ThrPro---......---Leu---GlnSerLeuTyr---ThrArgSer   321
Z6     Ser---------ThrPro---......---Leu---Gln---LeuTyr---ThrArgGly   322
Z3     ------------GlnSer---ArgIle---------LysVal---TyrAlaLys---Gly   319
Z321   Ser------------Ser---......------------------PheAlaThr---Asp   321
JY1    Ser---------ThrPro---......---Leu---Gln---LeuTyr---ThrArg...   332
```

```
                                       D3 <-|  * [        ]
HXB2   ............IleGlyAsnMetArgGlnAlaHis|CysAsnIleSerArgAlaLysTrp    338
BRU    --------------------------------------|------------------------    343
MN     ---------Ile------ThrIle--------------|------------------------    342
SC     ---------Ile------AspIle--------------|------------------------    337
SF2    ---------Ile------AspIle---Lys--------|------------------Gln---    340
NY5    ---------Ile------AspIle--------------|------------Lys---------    332
CDC4   ---------IleLeu------Ile--------------|------------------Gln---    346
WMJ2   ---------Ile------IleIle--------------|------------------------    333
RF     ---------Ile------AspIle---Lys--------|------Leu---------Gln---    350
MAL    ---------IleVal---AspIle---Arg---Tyr|---Thr---AsnGluThrGlu---    341
ELI    Arg---SerIle------.........-----------|------------------Gln---    337
Z6     ArgThrLysIle------.........-----------|------------LysGluAsp---    339
Z3     ---------IleThr---.........-----------|---------ThrAspGlyGlu---    333
Z321   ---------Ile------AspIle--------------|------Val------ThrGlu---    338
JY1    ---------IleLys---AspIle---------Tyr|------------Ala---Ala---    349
```

FIG. 2-7B

```
       [         ]                                          [
HXB2   AsnAsnThrLeuLysGlnIleAspSerLysLeuArgGluGlnPheGlyAsnAsn...Lys   357
BRU    ---Ala---------------Ala------------------------------------   362
MN     ---Asp------Arg------Val---------Lys---------Lys---...------   360
SC     ---------------------ValIle---------Asp------Glu---...------   355
SF2    ------------Glu------ValLys---------------------------------   359
NY5    ---Asp---------------ValThr------Lys---------Arg---...------   350
CDC4   ------------Gln------AlaThrThr-----------------------...------   364
WMJ2   ---------------------ValGlu------------------Lys---...------   351
RF     ------------------ValValThr------------------Asp---...------   368
MAL    AspLys------Gln---ValAlaVal------GlySerLeuLeuAsnLysThr---...   359
ELI    SerLys------Gln---ValAlaArg------GlyThrLeuLeuAsnLysThr---...   355
Z6     ---Lys------GlnArgValAlaIle------GlyAsnLeuLeuAsnLysThr---...   357
Z3     Arg---------Gln---ValAlaIleAla------Arg------Asn---...------   351
Z321   ---Asp------SerLysValAlaAlaGln------LysHis---Val---ThrSerThr   358
JY1    ---Lys------Gln---ValAlaLys------GlyAspLeuLeuAsnGlnThr---...   367
```

FIG. 2-7C

```
              ]           ┌─► C3
HXB2   ThrIleIlePheLysGln│SerSerGlyGlyAspProGluIleValThrHisSerPheAsn    377
BRU    ------------------│------------------------------------------    382
MN     ------Val---Asn---│-----------------------------Met------------    380
SC     ------------AsnArg│-----------------------------Met------------    375
SF2    ------Val---Asn---│-----------------------------Met------------    379
NY5    ------Val---Asn---│-----------------------------Met------------    370
CDC4   ------Ala---Asn---│-----------------------------Met------------    384
WMJ2   ------Val---AsnHis│------------------------------------------    371
RF     ------Val---ThrSer│-----------------------------Leu------------    388
MAL    Lys---------AsnSer│------------------------Thr---------------    379
ELI    Ile---Lys------Pro│------------------------Thr---------------    375
Z6     ---------------Pro│---------------Ala------Thr---------------    377
Z3     Ser---------AsnSer│---------------Ile------Thr------Thr------    371
Z321   Asp---------AlaAsn│---------------Val------Thr---------------    378
JY1    ---------------Pro│ProAla------------------Thr---------------    387
```

FIG. 2-8A

```
                                     C3 <---|---> D4
        *                             *  [        ]          [        ]
HXB2  CysGlyGlyGluPhePheTyrCys|AsnSerThrGlnLeuPheAsnSerThrTrp......  395
BRU   ------------------------|----------------------------------  400
MN    ------------------------|---ThrSerPro-----------------------  398
SC    ------------------------|------------------Ser--------------  393
SF2   ---Arg------------------|---Thr---------------Asn-----------  397
NY5   ------------------------|LysThr-----------------------------  388
CDC4  ------------------------|---------------------Ala------Asn  403
WMJ2  ------------------------|----------------------------------  389
RF    ------------------------|---Thr----------------------------  406
MAL   ---Arg------------------|---ThrSerLys-----------------------  397
ELI   ------------------------|---ThrSerGly-----------------------  393
Z6    ------------------------|---ThrSerGly-----------------------  395
Z3    ------------------------|---ThrSerGlu-----ThrGlyIle---------  389
Z321  ------------------------|---ThrSerGly--------Gly------LeuAsn  398
JY1   ------------------------|---ThrSerArg-----------------------  405
```

FIG. 2-8B

```
                  [        ]                                  [        ]
HXB2   ......PheAsnSerThr...TrpSerThr.........GluGlySerAsnAsnThrGlu   409
BRU    ------------------------------------------------------------   414
MN     ---AsnGly---Asn---------AsnAsnThrThr---...------------Asn...   413
SC     ------...---Gly------.........---------------------------Gly   403
SF2    ---ArgLeu---His------.........---------------.........---Lys   406
NY5    ---Leu------------------AsnAsp------Thr---Arg---.........Asp   401
CDC4   ValThrSer---Gly---------......---------......---ValThrArgLys   415
WMJ2   ------...---Gly---Asp.........------IleLys---Asp---LysAsn...   400
RF     ------...------------.........---------------------------Gly   416
MAL    ---GlnAsn---GlyAlaArg.........---------...Leu------Ser------   409
ELI    ------...---IleSerAla---AsnAsnIleThr---...Glu---------SerThr   408
Z6     ---AsnIle---AsnSer---.........---------------Ala---Ser------   407
Z3     ------...---Gly---------AspLysAsnCys---.........ThrSer------   401
Z321   GlyThrSer---Asn---------LysIleAspThr---.....................   409
JY1    ------...------SerThr---AsnAsnAspThr---.........Leu---Ser---   418
```

FIG. 2-8C

FIG. 2-9A

```
                                              D4 <--|--> C4
                                                    *
HXB2  GlySerAspThr.........IleThrLeuProCysArgIleLysGlnIleIleAsnMet   426
BRU   ------------------------------------------------Phe--------   431
MN    ............-----------------Gln---Lys---------------------   426
SC    ---Asn-----------------------------------Glu---------------   420
SF2   ---Asn---------------Ile-----------------------------------   423
NY5   AsnAsnGlu------------IleIle----------------------------Ser   418
CDC4  GlnLys------GlyAspIle----------------------------------Arg   435
WMJ2  ......Ser---Leu--------------------------------------------   416
RF    ---Asn------------------------------------------Val------   433
MAL   SerThrGlySer-----------------------------------------------   426
ELI   AsnThrAsn...-----------------Gln------------------Lys---   424
Z6    SerAspAsnLysLeu--------------Gln---------------------------   425
Z3    SerAsnCys---GlyAsn---------------------------ValValArgThr   420
Z321  ValAsn---------------Ile--------------------------Val------   426
JY1   ---Thr......---------Lys-----------------------------------   433
```

```
            --- CD4 binding region ---                           *
HXB2  TrpGlnLysValGlyLysAlaMetTyrAlaProProIleSerGlyGlnIleArgCysSer    446
BRU   ------Glu-------------------------------------------------------   451
MN    ------Glu--------------------------------Glu--------------------   446
SC    ------Glu--------------------------------Lys------ValLys-------   440
SF2   ------Glu--------------------------------Gly---------Ser-------   443
NY5   GlyArg---+++                                                      422
CDC4  ------Val---------------------Leu------Lys---Leu---------------   455
WMJ2  ------Gly--------------------------------Gln--------------------   436
RF    ------Glu----------------------------------------------Lys---Ile  453
MAL   ---------Thr-----------------------------Ala---Val---Asn---Leu   446
ELI   ValAlaGlyArg...------Ile-----------------GluArgAsn---Leu-------   443
Z6    ------Gly--------------------------------Glu---------Asn-------   445
Z3    ------Gly------Gln-----------------------Glu---Thr-------------   440
Z321  ------Arg------Gln-----------------------Lys---Val---Lys---Val   446
JY1   ------Gly--------------------------------Glu---Leu---Lys---Thr   453
```

FIG. 2-9B

```
              [         ]                                         C4 <--|--> D5
HXB2   SerAsnIleThrGlyLeuLeuLeuThrArgAspGlyGly.............|...AsnSer      461
BRU    ----------------------------------------------------|-------Asn     466
MN     -------------------------------------------------Lys|AspThrAsp      463
SC     ----------------------------------------AsnSerLysAsn|GlySerLys      460
SF2    ----------------------------------------------------|Thr---Val      459
NY5
CDC4   ----------------------------------------Gly---------|----...Glu     470
WMJ2   ----------------------------------------AsnSer------|----......     451
RF     ----------------------------------------------------|GluAspThr      469
MAL    ---------------Ile----------------------AsnSer------|----SerAsp     463
ELI    ----------------------------------------------------|----IleAsn     458
Z6     ----------------------------------------------------|----ThrAsn     460
Z3     ----------------------------------------AsnGlyLysCys|AsnSerLys      460
Z321   ------------Ile----------------------ValGly---------|----...Asn     461
JY1    ----------------------------------------Val---------|----...Asn     468
```

FIG. 2-9C

```
                         D5 <-|           |-> C5
             [            ]
HXB2  AsnAsnGluSer|GluIle|PheArgLeuGlyGlyGlyAspMetArgAspAsnTrpArgSer   481
BRU   ------Gly---|------|------Pro---------------------------------   486
MN    Thr---AspThr|------|------Pro---------------------------------   483
SC    ---GluAsnThr|------|------Pro---------------------------------   480
SF2   Thr---AspThr|---Val|------Pro---------------------------------   479
NY5
CDC4  ---GlnThrThr|------|------Pro---------------------------------   490
WMJ2  SerSerArgGlu|------|------Pro---------Asn---------------------   471
RF    Thr---ThrThr|------|------------------Asn---------------------   489
MAL   ---SerAspAsn|---Thr|Leu---Pro---------------------------Ile---   483
ELI   ---SerThrAsn|---Thr|------Pro---------------------------------   478
Z6    ---SerSerAsn|---Thr|------Pro---------------------------------   480
Z3    +++                                                              461
Z321  ---ThrSerAsn|---Thr|------Pro---------------------------------   481
JY1   ---SerThrAsn|---Thr|------Pro---------------Lys-------------Asn  488
```

FIG. 2-10A

```
HXB2   GluLeuTyrLysTyrLysValValLysIleGluProLeuGlyValAlaProThrLysAla   501
BRU    ------------------------------------------------------------   506
MN     ------------------------Thr---------------------------------   503
SC     ------------------------------------------------------------   500
SF2    ---------------------Ile------------------Ile---------------   499
NY5
CDC4   ------------------------------------------------------------   510
WMJ2   ------------------------Arg---------------------------------   491
RF     ------------------------Arg---------------------------Arg---   509
MAL    ------------------------Arg---------------------------------   503
ELI    ------------------------Gln---------------------------Arg---   498
Z6     ------------------------------------------------------------   500
Z3
Z321   ------------------------------------------------------------   501
JY1    ------------------------Arg---------------Ile---------Arg---   508
```

FIG. 2-10B

```
                          gp120 C5 <----|----> gp160 C5   (TO END)
                                gp120  \/  gp41
HXB2  LysArgArgValValGlnArgGluLysArgAlaValGly...IleGlyAlaLeuPheLeu   520
BRU   ----------------------------------------------------------     525
MN    ---------------------------------------Ala...-----------       521
SC    -------------------------------------------Thr--------Met---   520
SF2   -------------------------------------------IleVal-----Met---   519
NY5
CDC4  -------------------------------------------MetLeu-----Met---   530
WMJ2  -------------------------------------------Thr--------Met---   511
RF    -------------------------------------------Thr--------Met---   529
MAL   ---------------Glu----------------Ile------Leu-------Met---    522
ELI   ---------------Glu----------------Ile------Leu-------Met---    517
Z6    ---------------Glu----------------Ile------Leu-------Met---    519
Z3
Z321  ---------------Ala----------------Ile---Met...-------Phe---    520
JY1   ---------------Glu----------------Ile------Leu-------Val---    527
```

FIG. 2-10C

```
HXB2   GlyPheLeuGlyAlaAlaGlySerThrMetGlyAlaAlaSerMetThrLeuThrValGln    54Ø
BRU    ------------------------------------Arg---------------------    545
MN     ------------------------------------------Val---------------    541
SC     ------------------------------------Thr---------------------    54Ø
SF2    ------------------------------------Val---Leu---------------    539
NY5
CDC4   ------------------------------------Thr------Ala------------    55Ø
WMJ2   ------------------------------------Gly---Leu---------------    531
RF     ------------------------------------Gly---Ile---------------    549
MAL    ------------------------------------------Leu---------------    542
ELI    ------------------------------------Arg---Val---------------    537
Z6     ------------------------------------------Val---------------    539
Z3
Z321   ------------------------------------------Ile---------------    54Ø
JY1    ------------------------------------Val---ValAla------Gly---    547
```

FIG. 2-11A

HXB2	AlaArgGlnLeuLeuSerGlyIleValGlnGlnGlnAsnAsnLeuLeuArgAlaIleGlu	560
BRU	--	565
MN	------Leu---	561
SC	------Leu---	560
SF2	--	559
NY5		
CDC4	---Lys	570
WMJ2	---Asp	551
RF	------His---	569
MAL	--	562
ELI	------------Met---	557
Z6	------------Met---	559
Z3		
Z321	------Arg---	560
JY1	--	567

FIG. 2-11B

```
HXB2  AlaGlnGlnHisLeuLeuGlnLeuThrValTrpGlyIleLysGlnLeuGlnAlaArgIle   580
BRU   ------------------------------------------------------------   585
MN    ------------Met-------------------------------------------Val   581
SC    ----------------------------------------------------------Val   580
SF2   ----------------------------------------------------------Val   579
NY5
CDC4  ------------------------------------------------------------   590
WMJ2  ----------------------------------------------------------Val   571
RF    ----------------------------------------------------------Val   589
MAL   ----------------------------------------------------------Val   582
ELI   ------------------------------------------------------------   577
Z6    ------------------------------------------------------------   579
Z3
Z321  ------------------Lys---------------------------------------   580
JY1   ------------Met-------------------------------------------Val   587
```

FIG. 2-11C

```
                                                                  *
HXB2   LeuAlaValGluArgTyrLeuLysAspGlnGlnLeuLeuGlyIleTrpGlyCysSerGly   600
BRU    ------------------------------------------------------------   605
MN     ---------------------------------------------Phe------------   601
SC     ------------------------Arg---------------------------------   600
SF2    ------------------------Arg---------------------------------   599
NY5
CDC4   ---------------------------------------------Phe------------   610
WMJ2   ------------------------Arg---------------------------------   591
RF     ------------------------Arg---------------------------------   609
MAL    ------------------------Gln---------Arg---------Met---------   602
ELI    ------------------------------------------------------------   597
Z6     ------------------------------------------------------------   599
Z3
Z321   ------------------------------------------------------------   600
JY1    ------------Ser---------------------------------------------   607
```

FIG. 2-12 A

```
                        *                          [         ]       [        ]
HXB2   LysLeuIleCysThrThrAlaValProTrpAsnAlaSerTrpSerAsnLysSerLeuGlu    620
BRU    ------------------------------------------------------------    625
MN     ------------------Thr------------------------------------Asp    621
SC     ------------------Thr------------Thr---------------------Asp    620
SF2    ------------------------------------------------------------    619
NY5
CDC4   ------------------------------------------------------Thr---Asp    630
WMJ2   ------------------Thr-----------------------------------MN  611
RF     ------------------Thr------------------------------------Asn    629
MAL    ---His------------Phe------------Ser------------Arg------Asp    622
ELI    ---His------------Asn------------Ser------------Arg------Asn    617
Z6     ------------------Thr------------Ser------------Arg------Asn    619
Z3
Z321   ---Ile------Pro---Asn------------Ser------------------GlnSer    620
JY1    ---His------------Thr------------Ser------------------------    627
```

FIG. 2-12 B

```
                       [         ]                                          [         ]
HXB2   GlnIleTrpAsnHisThrThrTrpMetGluTrpAspArgGluIleAsnAsnTyrThrSer   640
BRU    ------------AsnMet------------------------------------------   645
MN     Asp---------AsnMet---------Gln---Glu---------Asp------------   641
SC     Lys------GlyAsnMet---------------Glu---------Asp------------   640
SF2    Asp------AspAsnMet---------Gln---Glu---------Asp---------Asn   639
NY5
CDC4   ------------AsnMet---------------------------Asp---------His   650
WMJ2   ---------AspAsnLeu---------------Glu---------Asp------------   631
RF     Met---------AsnMet---------Gln---Glu---------Asp---------Gly   649
MAL    Asp---------AsnMet---------Gln---GluLys------Ser---------Gly   642
ELI    Glu------GlnAsnMet---------------Glu---------Asp---------Gly   637
Z6     Asp------GlnAsnMet---------------Glu---------Asp---------Gly   639
Z3
Z321   Asp------AspLysMet------Leu---------Lys---ValSer---------Gln   640
JY1    Glu---------AsnMet------Ile------Glu---------Asp---------Gly   647
```

FIG. 2-12C

```
HXB2  LeuIleHisSerLeuIleGluGluSerGlnAsnGlnGlnGluLysAsnGluGlnGluLeu  660
BRU   ------------------------------------------------------------  665
MN    ------Tyr------Leu---Lys------Thr---------------------------  661
SC    ------TyrThr------------------------------------------------  660
SF2   Thr---TyrThr---Leu------------------------------------------  659
NY5
CDC4  ------TyrThr---------------------------------Gln---------  670
WMJ2  Ile---Tyr---------------------------Gly------------------  651
RF    Ile---TyrAsn---Leu------------------------------------------  669
MAL   Ile---TyrAsn------------------Ile------------------Lys------  662
ELI   ------Tyr---------------------Thr------------------Lys------  657
Z6    ------TyrArg------------------Thr---------------------------  659
Z3
Z321  Val---TyrAsn------------------Thr---------Ile------ArgAsp---  660
JY1   Val---Tyr------------Asn------Ile---------------------Asp---  667
```

FIG. 2-13A

```
                 [           ]
HXB2  LeuGluLeuAspLysTrpAlaSerLeuTrpAsnTrpPheAsnIleThrAsnTrpLeuTrp   680
BRU   ------------------------------------------------------------   685
MN    ---------------------------------------Asp------------------   681
SC    ------------------------------------------------------------   680
SF2   ---------------------------------------Ser------------------   679
NY5
CDC4  ---Gln---------------------------Thr---SerAsp------Lys------   690
WMJ2  ---------------------------------------Asp------------------   671
RF    ------------------------Asn------------Asp------Gln---------   689
MAL   ---------------------------------------Ser---SerLys---------   682
ELI   ---------------------------------------Ser------Gln---------   677
Z6    ------------------------------------------------Gln---------   679
Z3
Z321  ---Ala------------------Asn------------Asp---Ser------------   680
JY1   ---Gln---------------------------------Ser------Lys---------   687
```

FIG. 2-13B

```
HXB2   TyrIleLysLeuPheIleMetIleValGlyGlyLeuValGlyLeuArgIleValPheAla  700
BRU    ---------Ile-----------------------------------------------  705
MN     ---------Ile-----------------------------------------------  701
SC     ---------Ile--------------------------------------------Thr  700
SF2    ---------Ile-----------------------------------------------  699
NY5
CDC4   ---------Ile------------------------Ile--------------------  710
WMJ2   ---------Ile------------------------Ile-----------------Thr  691
RF     ------ArgIle-------------------------------Lys-------------  709
MAL    ------ArgIle------IleVal------------Ile----------Ile-------  702
ELI    ---------Ile------------Ile---------Ile--------------------  697
Z6     ---------Ile------------------------Ile--------------------  699
Z3
Z321   ---------Ile------------------------Ile--------------------  700
JY1    ---------Ile------------------------Ile-----------------Thr  707
```

FIG. 2-13C

```
                                                                      \/ 3' sj
HXB2   ValLeuSerIleValAsnArgValArgGlnGlyTyrSerProLeuSerPheGlnThrHis    720
BRU    ------------------------------------------------------------    725
MN     ------------------------------------------------Leu------Arg    721
SC     ---------------------------------------------------------Arg    720
SF2    ---------------------------------------------------------Arg    719
NY5
CDC4   ---------------------------------------------------------Leu    730
WMJ2   ------------------------------------------------------------    711
RF     ------------------------------------------------------------    729
MAL    ---------Leu------------------------------------Leu------Leu    722
ELI    ---------Leu---------------------------------------------Leu    717
Z6     ---------Leu---------------------------------------------Leu    719
Z3
Z321   ------------Ile------------------------------------------Leu    720
JY1    ---------Leu---------------------------------------------Leu    727
```

FIG. 2-14A

```
                                                  <- tat cds end
HXB2   LeuProIleProArgGlyPro...AspArgProGluGlyIleGluGluGluGlyGlyGlu    739
BRU    ------Thr-------------------------------------------------------  744
MN     Pro---Val-------------------------------------------------------  740
SC     ------SerGln----------------------------------------------------  739
SF2    ------Val------------------------Asp----------------------------  738
NY5
CDC4   ------Asn------------------------------Thr------Gly----------  749
WMJ2   ------Thr-------------------------------------------------------  730
RF     ------Ala---------------------------------------Gly------------  748
MAL    ------Thr------------Pro----------------------------------------  742
ELI    ------Ala------------------------------Thr----------------------  736
Z6     ------Ala------Glu----------------------------------------------  738
Z3
Z321   ThrHisHisGln---Glu------------------Arg----------Gly----------  739
JY1    ------Ala-------------------------------------------------------  746
```

FIG. 2-14B

42/59

```
                                     [         ]
HXB2  ArgAspArgAspArgSerIleArgLeuValAsnGlySerLeuAlaLeuIleTrpAspAsp   759
BRU   ------------------------------------------------------------   764
MN    ------------Thr---Gly---------His---Phe------Ile------Val---   760
SC    ------------------Gly---------Asp---Phe------Ile------Val---   759
SF2   ------------------Val---------Asp---Phe---------------Glu---   758
NY5
CDC4  ---Gly------Gly---Thr---------His---Phe---------Val---------   769
WMJ2  ------------------Val---------His---Phe---------------------   750
RF    ------------------GlyGlyAla---------Phe---Thr---------------   768
MAL   GlnGly---Gly------------------------PheSer------------------   762
ELI   ---Gly------------Val------Leu------PheSer------------------   756
Z6    ---Gly------------------------------PheSer------------------   758
Z3
Z321  Gln---------------------------Ser---Phe---Pro---Ala---------   759
JY1   GlnGly------------------------------PheSer------Phe---------   766
```

FIG. 2-14C

SUBSTITUTE SHEET

```
                       *
HXB2  LeuArgSerLeuCysLeuPheSerTyrHisArgLeuArgAspLeuLeuLeuIleValThr   779
BRU   ------------------------------------------------------------   784
MN    ------------Phe---------------His...------------------AlaAla   779
SC    +++---------------------------------------------------------   779
SF2   ---------------------------Arg------------------------AlaAla   778
NY5
CDC4  ---------------------------------------------------------Ala   789
WMJ2  ---------------------------------------------------------Lys   77Ø
RF    ---TrpThr------Ser---------------------------------------Val   788
MAL   ------Asn---------------------------------------------Ala---   782
ELI   ---------------------------------------------Ile------AlaVal   776
Z6    ------Asn------------------------------------Ile------AlaAla   778
Z3
Z321  ---------------------Cys-----------------CysAla-------AlaAla   779
JY1   ------Asn------------------------------------Ile------Ala---   786
```

FIG. 2-15A

```
HXB2   ArgIleValGluLeuLeuGlyArgArgGlyTrpGluAlaLeuLysTyrTrpTrpAsnLeu   799
BRU    ------------------------------------------------------------   804
MN     ------------------------------------Val---------------------   799
SC     ------------------------------------------------------------   799
SF2    ---Thr------Ile------His--------------------------------Ser---   798
NY5
CDC4   ------------------------------------Val---------------------   809
WMJ2   ------------------------------------------------------------   790
RF     ------------------------------------------------------------   808
MAL    ---------------------------------------------Leu------------   802
ELI    ---------------------------------AspIle------Leu------------   796
Z6     ---------------------------------------------Leu------------   798
Z3
Z321   ------------Thr---Ile---------------Thr------LeuGly---------   799
JY1    ---------------------------------------Ile---Leu---Ser------   806
```

FIG. 2-15B

```
                                 <- trs/art cds end  [         ]
HXB2   LeuGlnTyrTrpSerGlnGluLeuLysAsnSerAlaValSerLeuLeuAsnAlaThrAla   819
BRU    ------------------------------------------------------------   824
MN     ---------------------------Ser------------------------------   819
SC     ------------------------Arg------------------PheVal---------   819
SF2    ------------Ile--------------------------Trp----------------   818
NY5
CDC4   ---------------------------------------------Val---Val------   829
WMJ2   ---------------Lys-----------------------Gly-----------Ile---   81Ø
RF     ---------------------------------------------------Thr------   828
MAL    ------------Gly-------------------------Ile------------Thr------   822
ELI    ------------------------Arg------------Ser------PheAsp---Ile---   816
Z6     ---------------Arg------Arg------------Ser---------AspThrIle---   818
Z3
Z321   ValIle------Gly-------------------------IleAsn---------AspThrVal---   819
JY1    ------------Thr--------------------PheIle-----------------------   826
```

FIG. 2-15C

```
                                                     *
HXB2  IleAlaValAlaGluGlyThrAspArgValIleGluValValGlnGlyAlaCysArgAla   839
BRU   ------------------------------------------------------------   844
MN    ---------------------------------------Leu---Arg---Gly------   839
SC    ------------------------------------LeuLeu---Arg---Phe------   839
SF2   ---------Thr---------------------------Ala---Arg---Tyr------   838
NY5
CDC4  ---------------------------------------------ArgIleTyr------   849
WMJ2  ---------------------------------------------ArgIle---------   830
RF    ---------------------------Ile---------Ala---ArgIleLeu------   848
MAL   ---------------Cys------------------IleGly---ArgPheGly------   842
ELI   ------------------------------------IleIle---Arg------------   836
Z6    ------------------------------------Ile---ArgArgThrTyr------   838
Z3
Z321  ------------AspTrp---------------------------Arg---Gly------   839
JY1   ---------------------------Ile------LeuIleArgArg---Phe------   846
```

FIG. 2-16A

```
HXB2   IleArgHisIleProArgArgIleArgGlnGlyLeuGluArgIleLeuLeu+++   857
BRU    -------------------------------------------------------   862
MN     ---Leu---------Thr-----------------------Ala---------   857
SC     ---Leu---------Thr-----------------------Ala---Gln---   857
SF2    ---Leu------His--------------------------Leu---------   856
NY5
CDC4   PheLeu---------------------------Phe------Ala---------   867
WMJ2   ---Ile-----------------------------------Ala---------   848
RF     PheLeu-----------------------------------Ala---------   866
MAL    ---Leu---------------------------Phe------Ala---------   860
ELI    ValLeuAsn--------------------------------Ser---------   854
Z6     ValLeuAsnVal---Thr-----------------------Leu---------   856
Z3
Z321   PheLeuAsn--------------------------------Ala---------   857
JY1    ValLeu---------------Val-----------------Ala---------   864
```

FIG. 2-16B

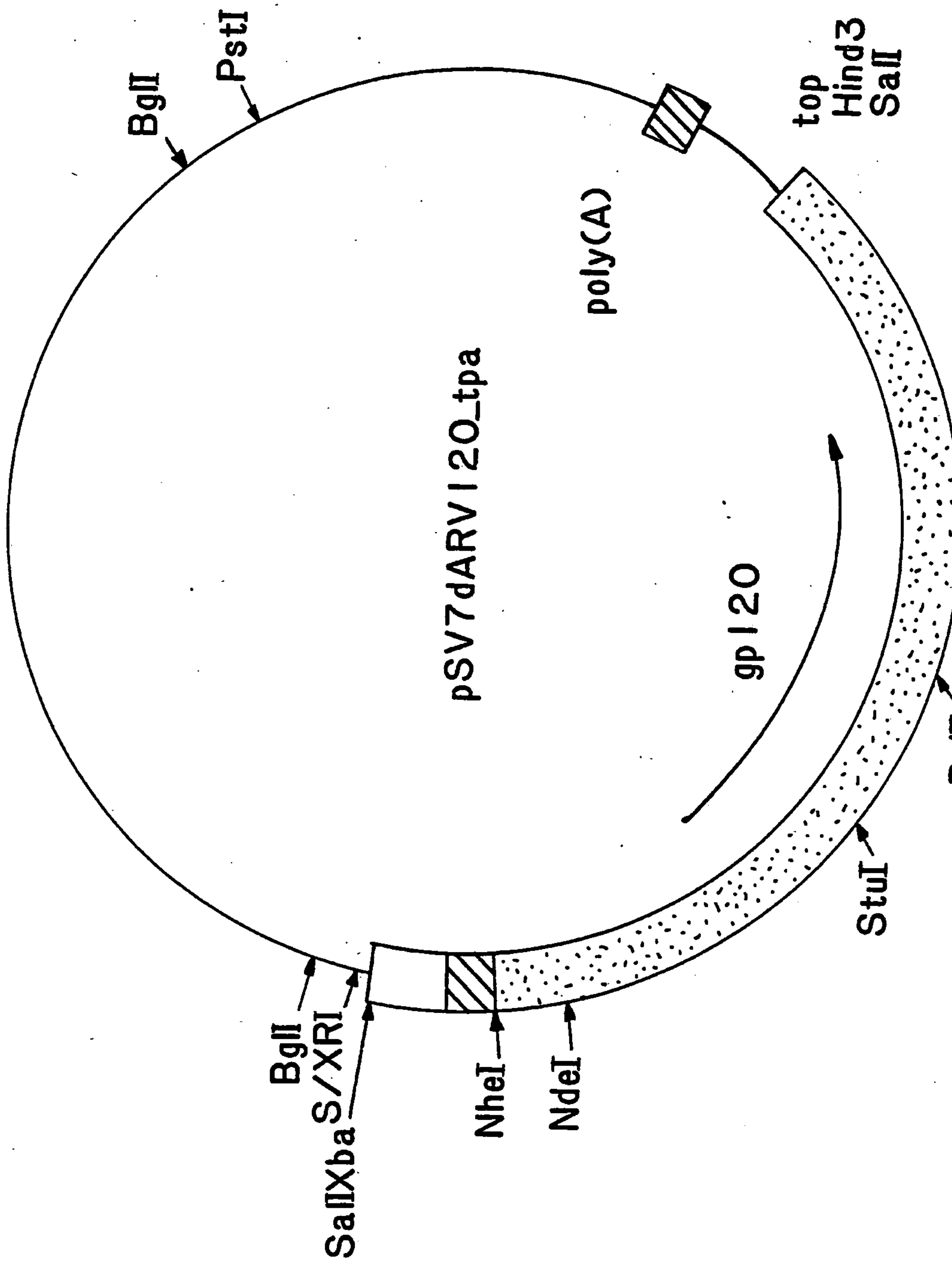

BglI
PstI
top
Hind3
SalI
poly(A)
pSV7dARV120_tpa
gp120
BglII
StuI
NdeI
NheI
SalIXba
S/XRI
BglI

FIG. 3

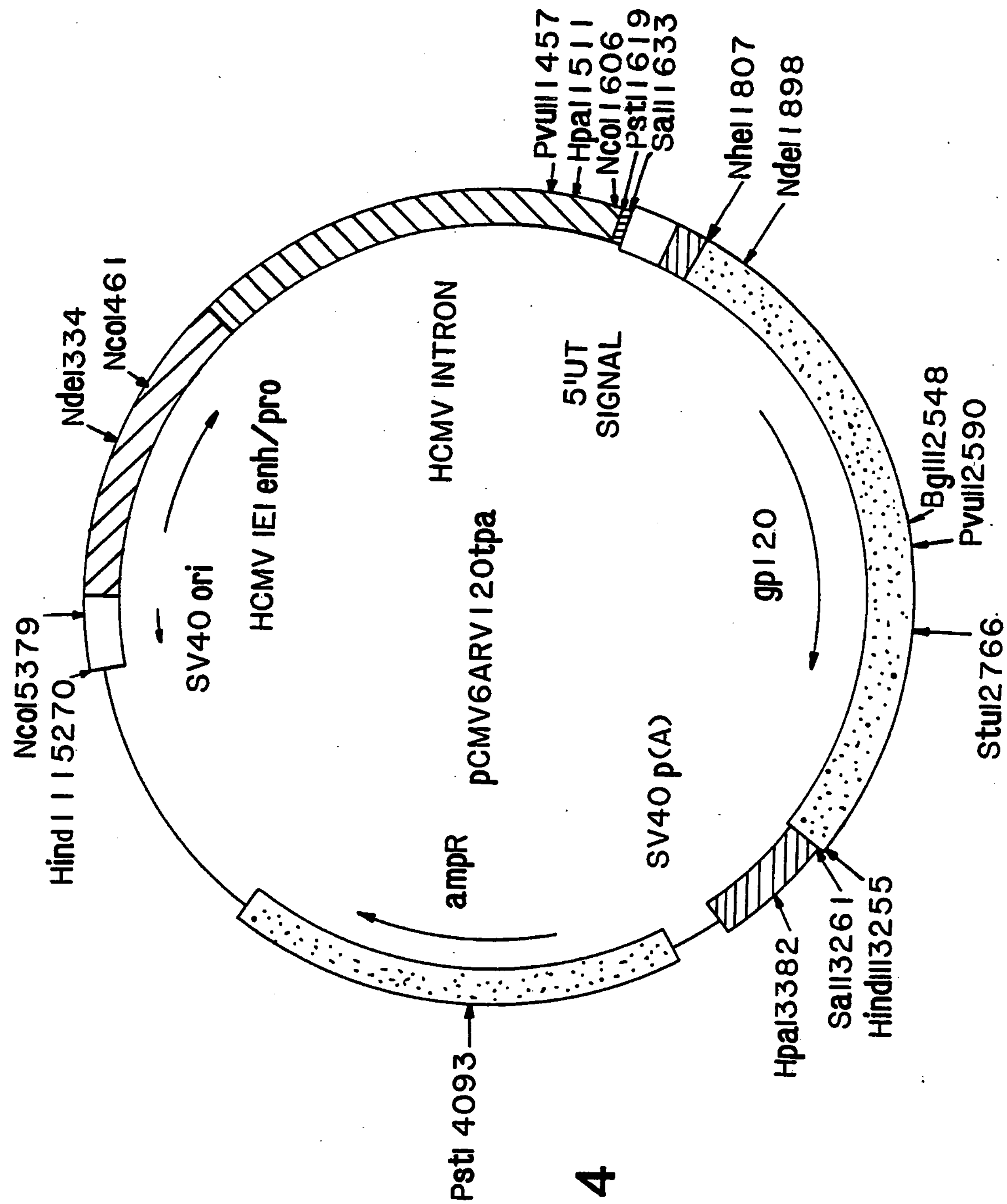

PvuII 1457
HpaI 1511
NcoI 1606
PstI 1619
SalI 1633
NheI 1807
NdeI 1898
NdeI334
NcoI461
BglII2548
PvuII2590
StuI2766
NcoI5379
HindIII 5270
HpaI3382
SalI3261
HindIII3255
PstI 4093
HCMV INTRON
5'UT
SIGNAL
HCMV IEI enh/pro
SV40 ori
pCMV6ARV120tpa
gp120
SV40 p(A)
ampR

FIG. 4

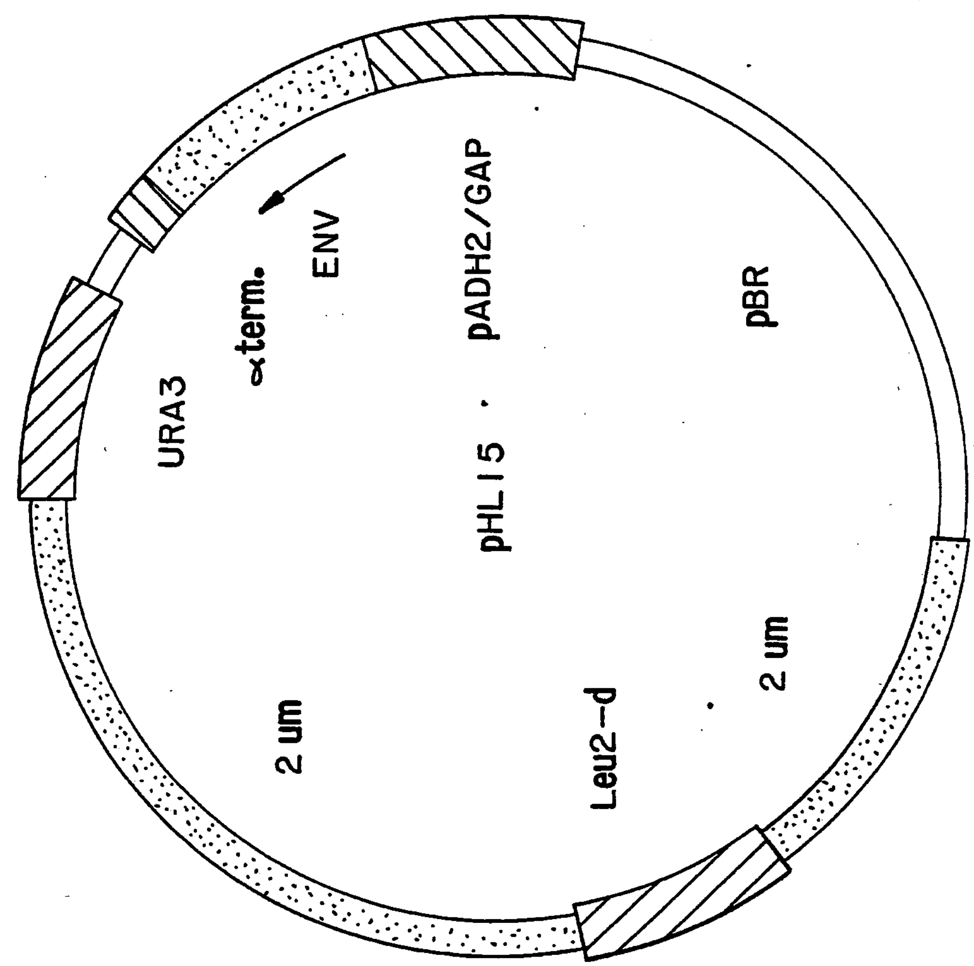


FIG. 5

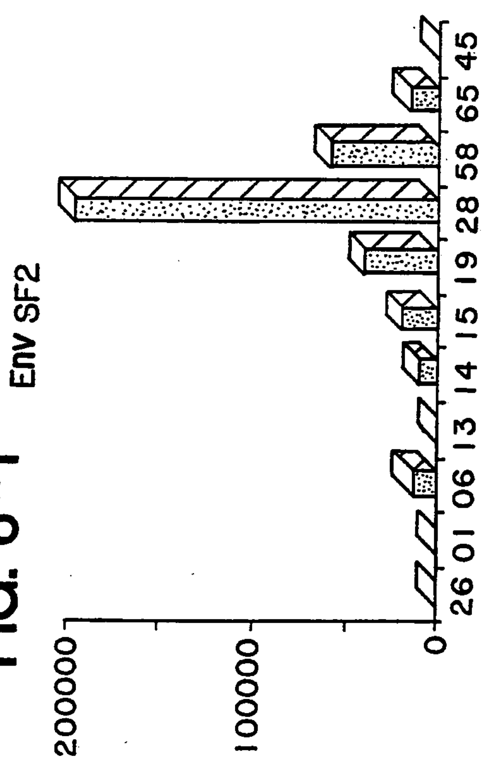


FIG. 6–1

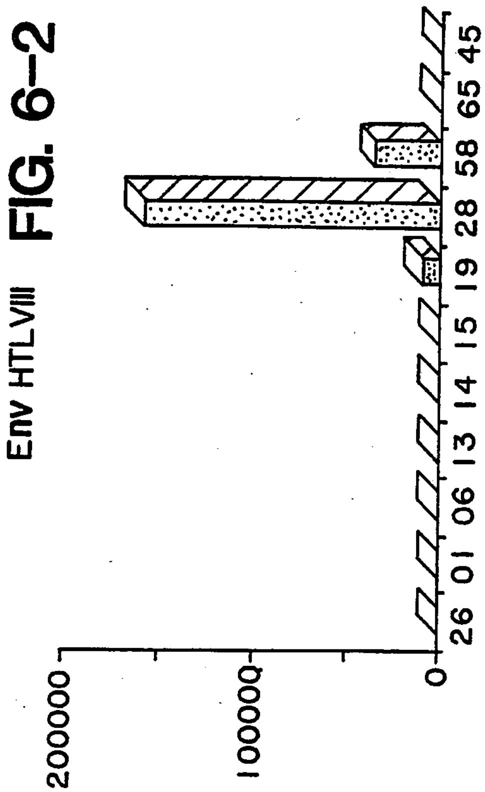


FIG. 6–2

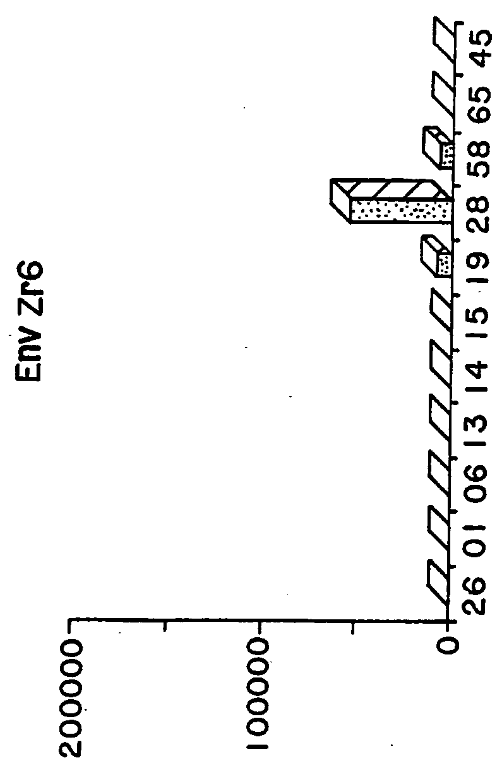


FIG. 6–3

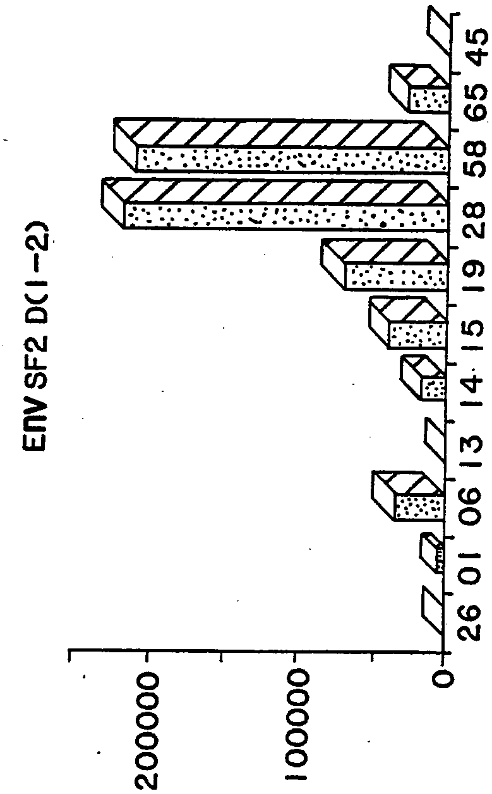


FIG. 6–4

FIG. 6-6 EnvSF2 D(3-5)

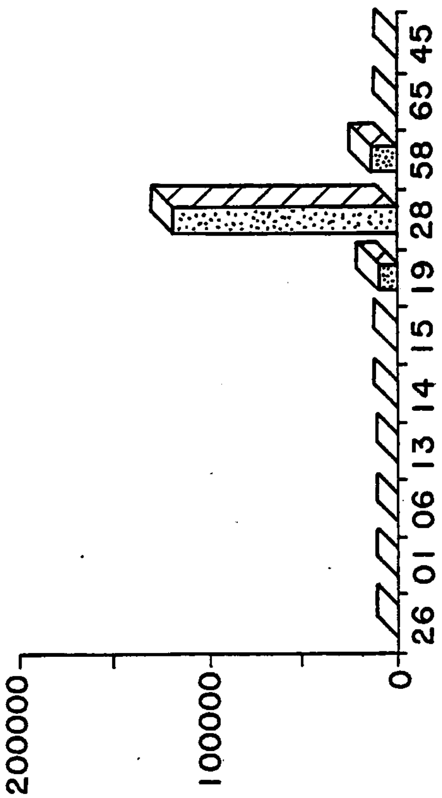


FIG. 6-5 Env SF2 D3E

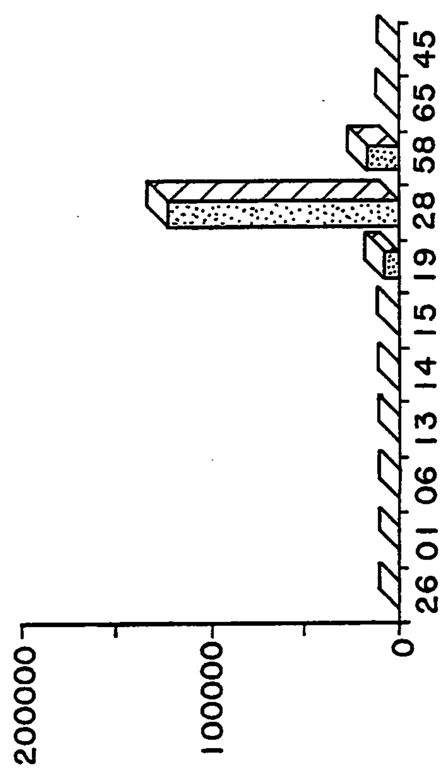


FIG. 6-7 Env SF2 D(1-5)

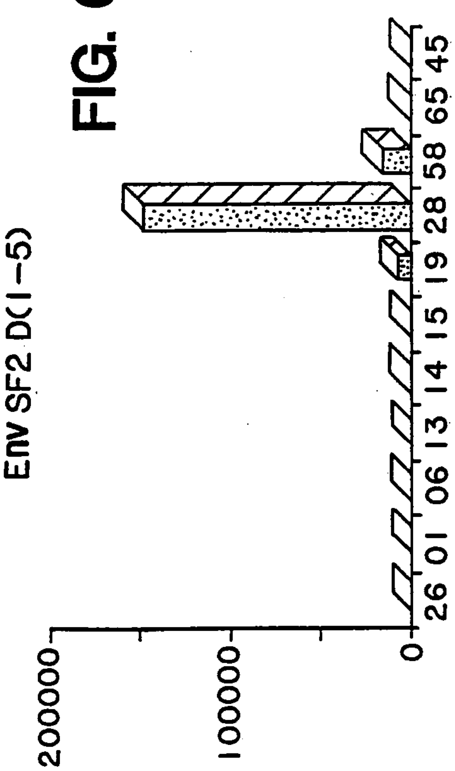


FIG. 7-1

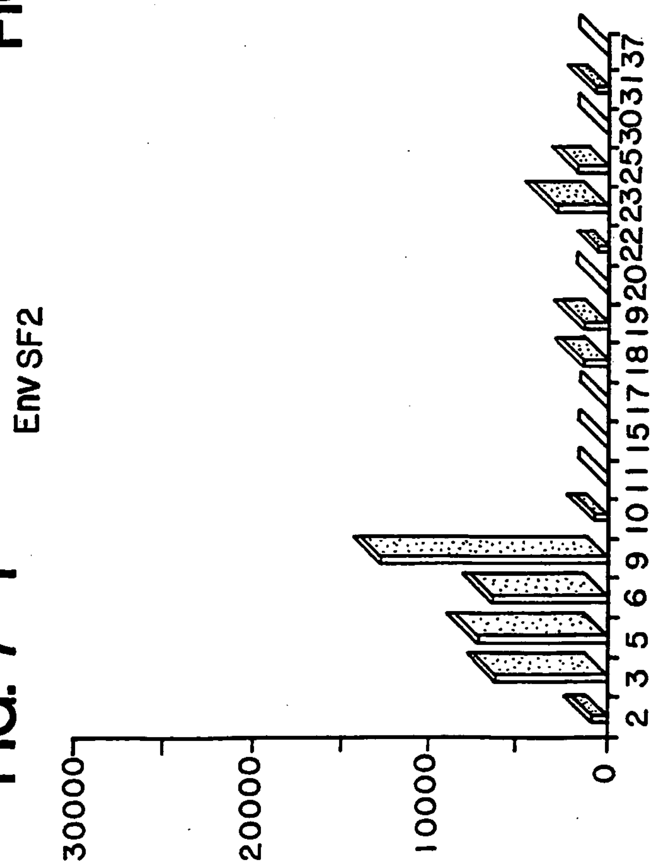
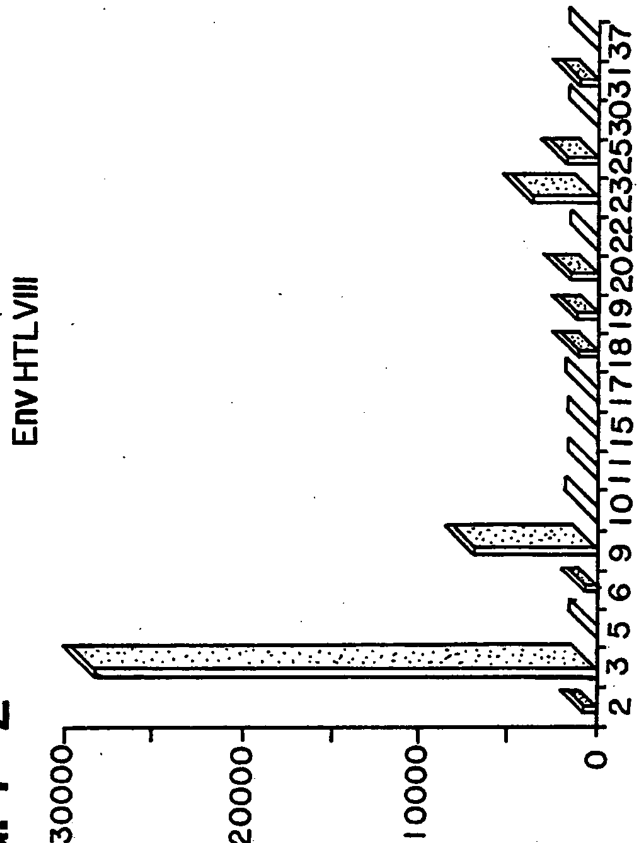

Env SF2
30000
20000
10000
0
2 3 5 6 9 10 11 15 17 18 19 20 22 23 25 30 31 37

FIG. 7-2


Env HTLV III
30000
20000
10000
0
2 3 5 6 9 10 11 15 17 18 19 20 22 23 25 30 31 37

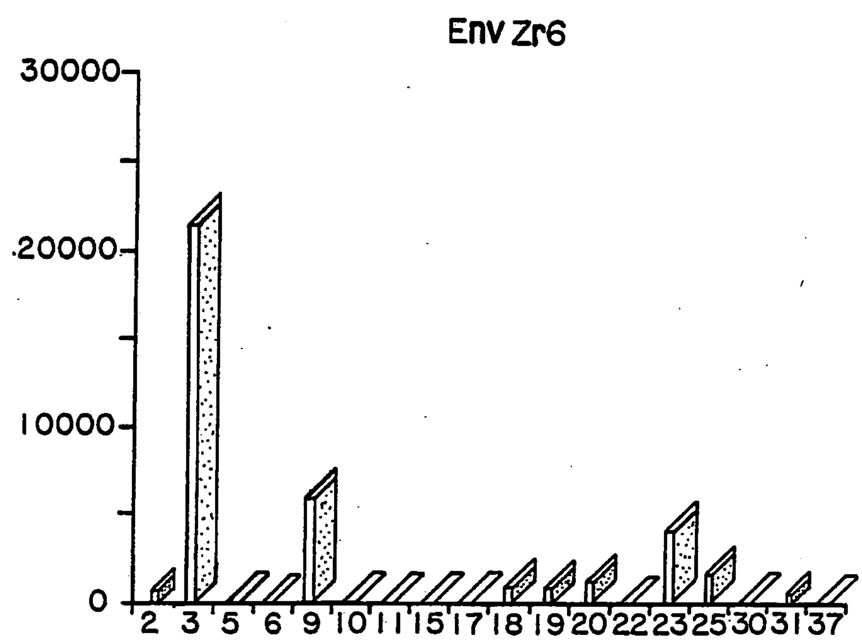

Env Zr6
30000
20000
10000
0
2 3 5 6 9 10 11 15 17 18 19 20 22 23 25 30 31 37

FIG. 7-3

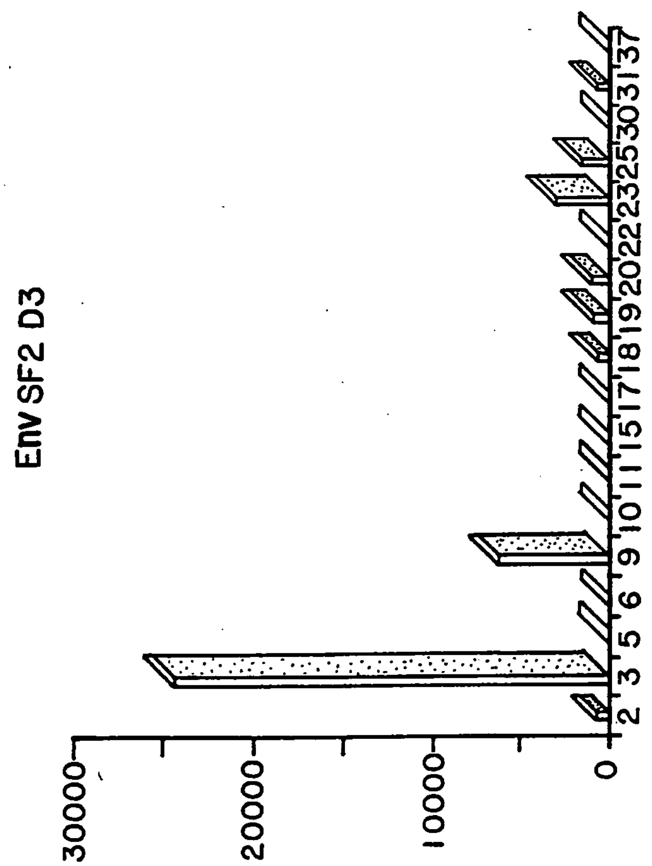

Env SF2 D3
30000
20000
10000
0
2
3
5
6
9
10
11
15
17
18
19
20
22
23
25
30
31
37

FIG. 7-4

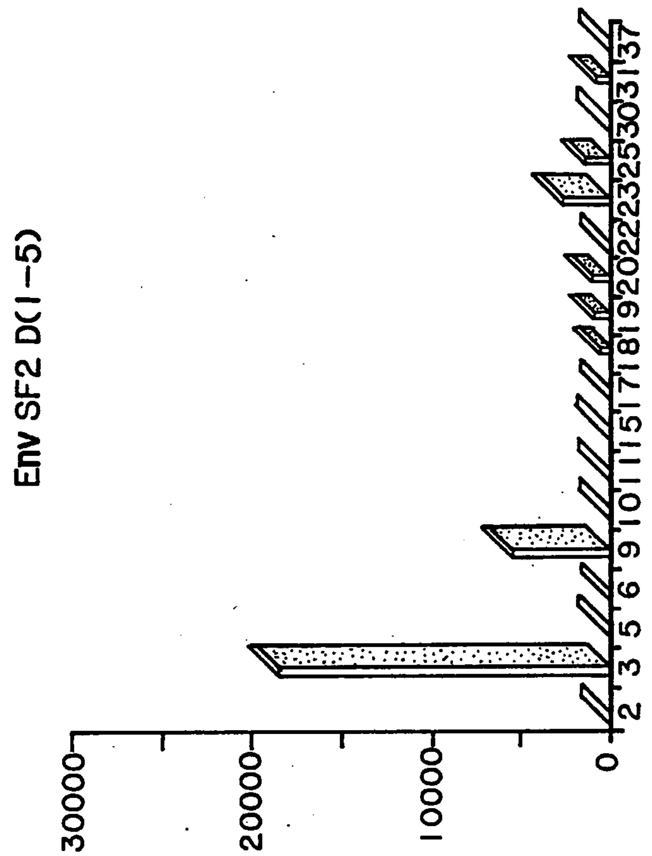

Env SF2 D(1-5)
30000
20000
10000
0
2
3
5
6
9
10
11
15
17
18
19
20
22
23
25
30
31
37

FIG. 7-5

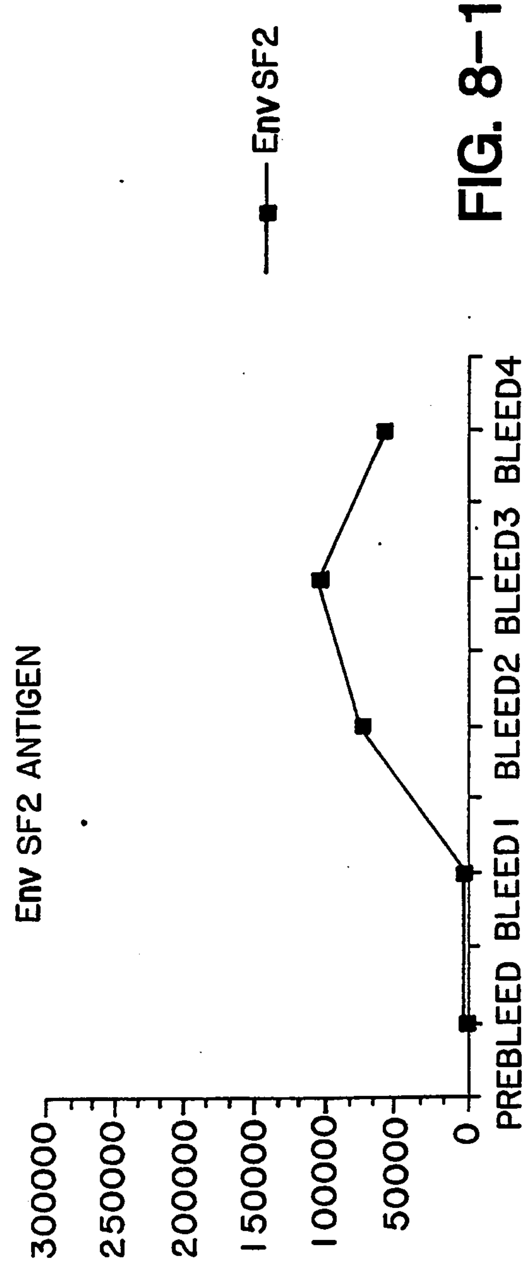

Env SF2 ANTIGEN
300000
250000
200000
150000
100000
50000
0
PREBLEED BLEED1 BLEED2 BLEED3 BLEED4
Env SF2

FIG. 8-1

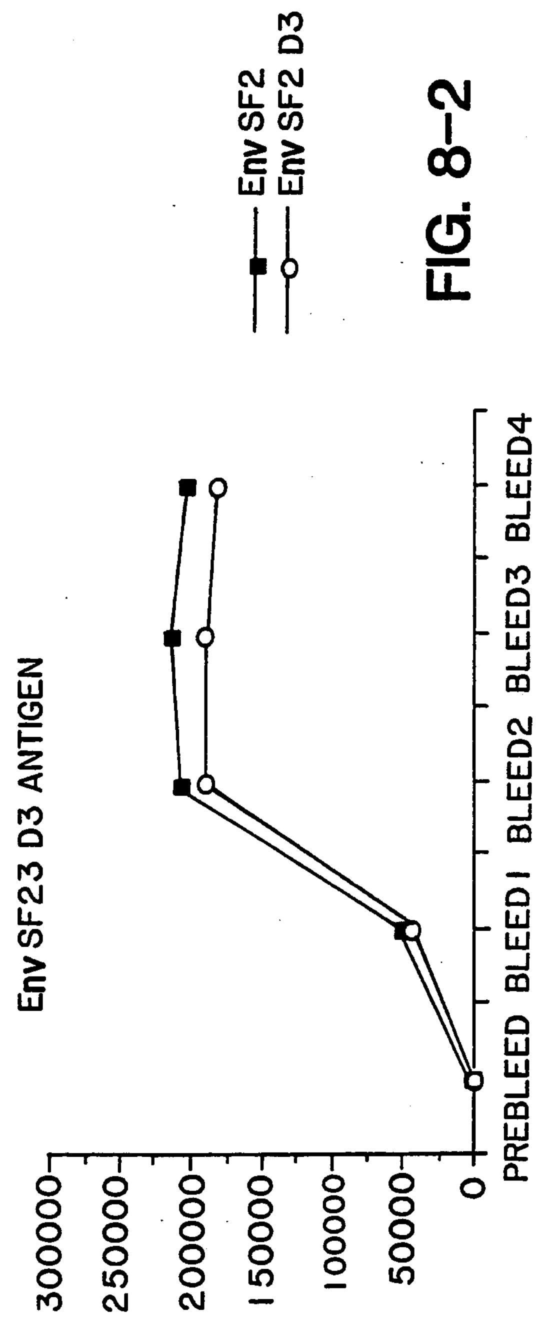

Env SF23 D3 ANTIGEN
300000
250000
200000
150000
100000
50000
0
PREBLEED BLEED1 BLEED2 BLEED3 BLEED4
Env SF2
Env SF2 D3

FIG. 8-2

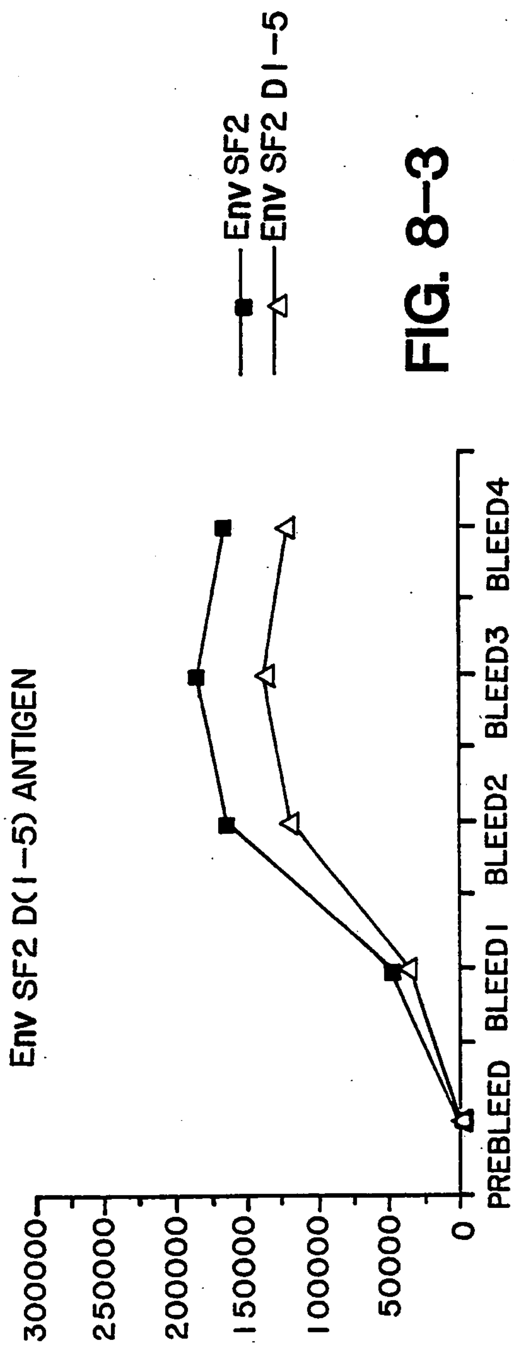

Env SF2 D(1-5) ANTIGEN
300000
250000
200000
150000
100000
50000
0
PREBLEED
BLEED1
BLEED2
BLEED3
BLEED4
Env SF2
Env SF2 D1-5

FIG. 8-3

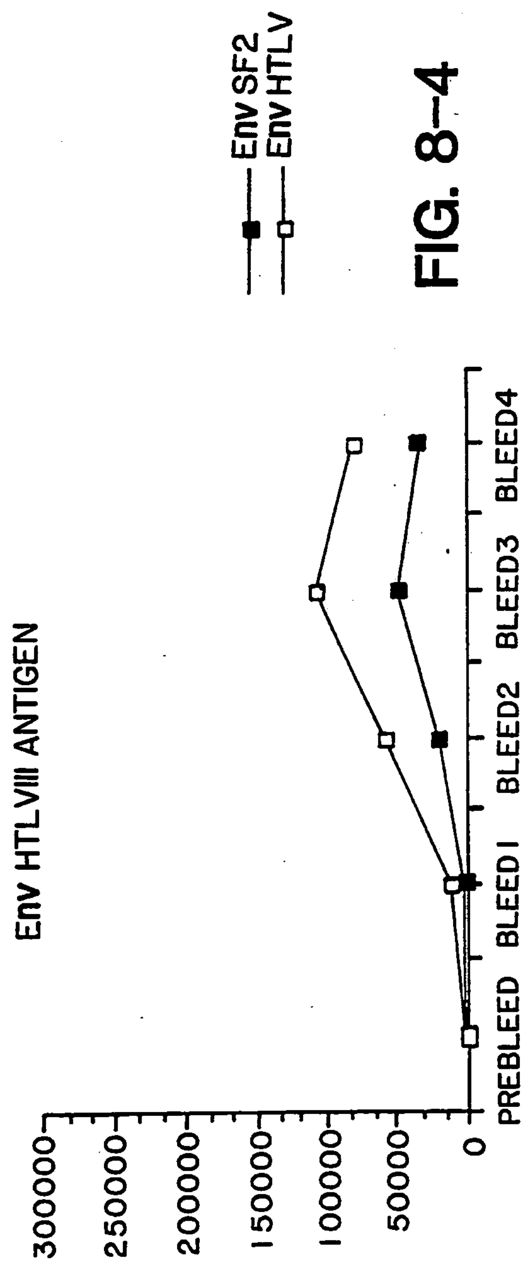
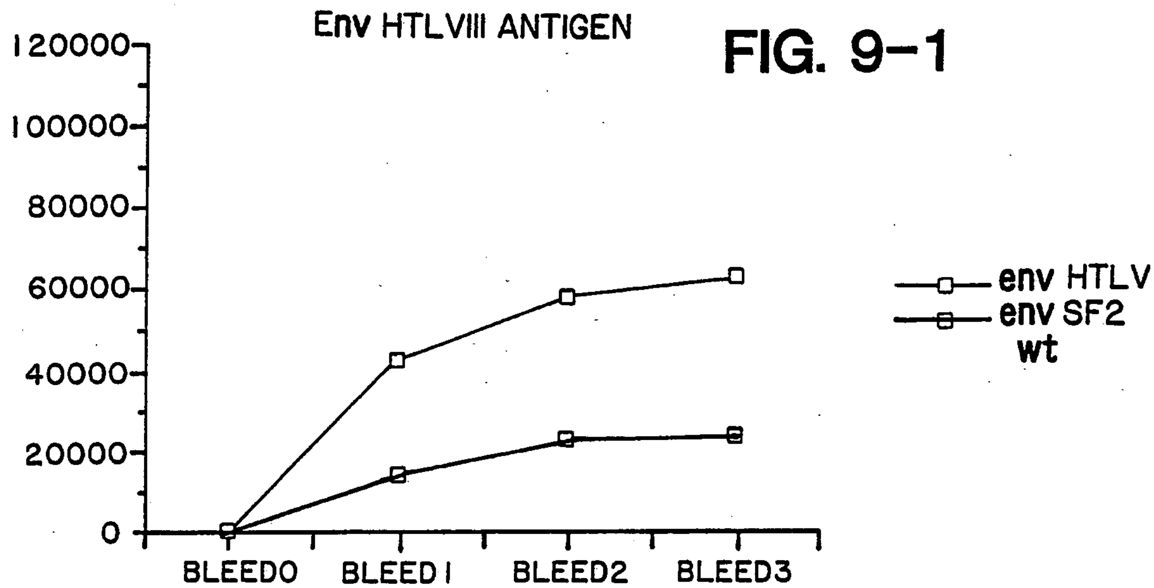
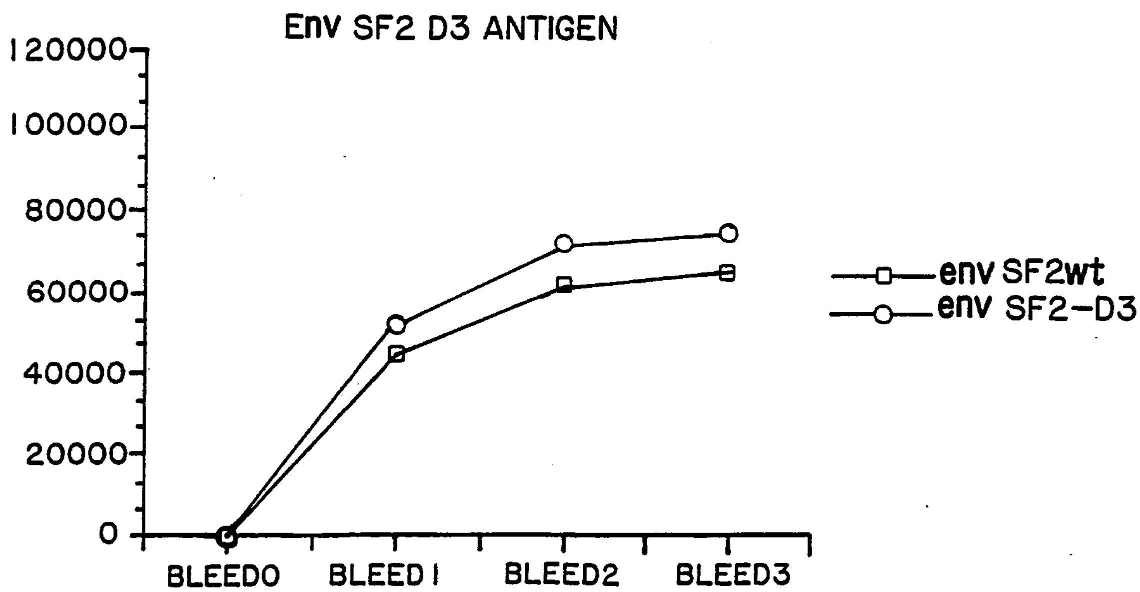

Env HTLVIII ANTIGEN
300000
250000
200000
150000
100000
50000
0
PREBLEED
BLEED1
BLEED2
BLEED3
BLEED4
Env SF2
Env HTLV

FIG. 8-4



Env HTLVIII ANTIGEN
120000
100000
80000
60000
40000
20000
0
BLEED0
BLEED1
BLEED2
BLEED3
env HTLV
env SF2 wt

FIG. 9-1



Env SF2 D3 ANTIGEN
120000
100000
80000
60000
40000
20000
0
BLEED0
BLEED1
BLEED2
BLEED3
env SF2wt
env SF2-D3

FIG. 9-2

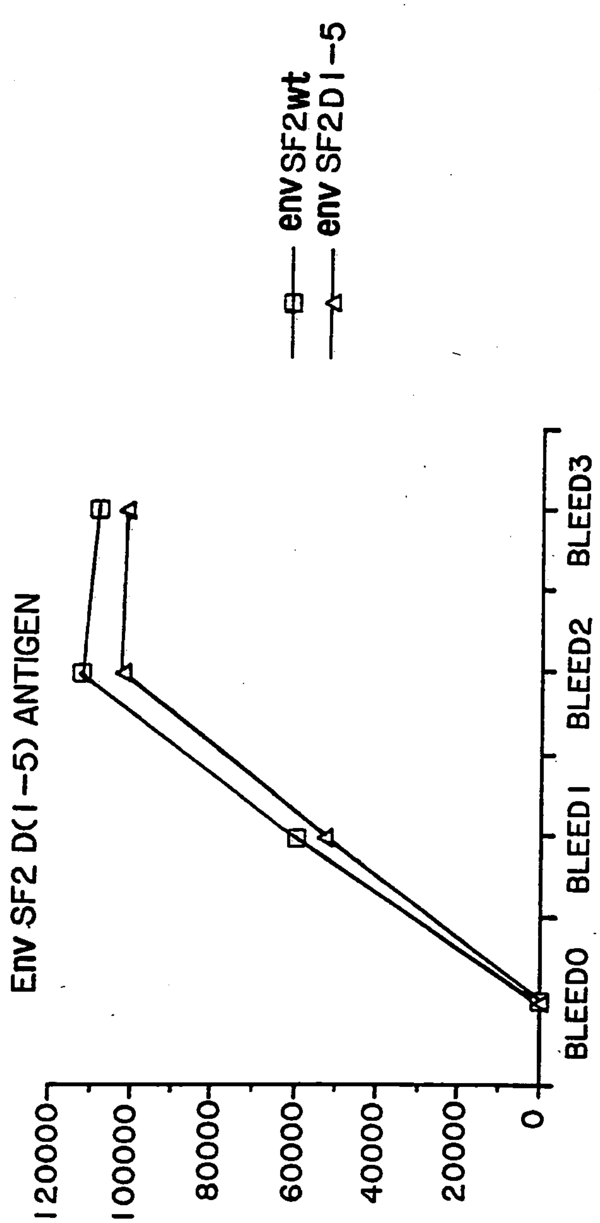

Env SF2 D(1 –5) ANTIGEN
envSF2wt
env SF2D1 –5
120000
100000
80000
60000
40000
20000
0
BLEED0
BLEED1
BLEED2
BLEED3

FIG. 9–3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03605

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K39/395; C12P21/02; C12Q1/70, C12N15/00
U.S. Cl.: 530/326; 435/5,7; 424/85.8, 88, 89; 435/172.3, 320

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
U.S.	530/326; 435/5,7; 424/85.8, 88, 89; 435/172.3, 320

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

Databases: Chemical Abstracts Services Online (File CA, 1967-1989; File Biosis, 1969-1989). Automated Patent System (File USPAT, 1975-1989). Protein databases: PIR, Swiss-Prot.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category*	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP 0187041, published 9 July 1986, (Capon), "Molecularly cloned acquired immunodeficiency syndrome polypeptides, intermediates therefor and methods and materials for their use."	1-35
X Y	Journal of Virology, Volume 61, No. 2, February 1987, (Modrow), "Computer-Assisted Analysis of Envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions", see pages 570-578.	1-26, 31-35 28-30
X Y	Vaccines 87, Cold Spring Harbor Laboratory, 1987, (Alizon), "Genetic variability of the human and simian aids virus", see pages 146-153.	1-30 31-35

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 December 1989	22 JAN 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	D. Bernstein D. Bernstein

Form PCT/ISA/210 (second sheet) (Rev.11-87)

International Application No. PCT/US89/03605

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ......, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers__________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☒ No required additional search fees were timely paid by the applicant; ~~Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:~~ however, the International Searching Authority has decided to search all groups corresponding to claims 1-35.
4. ☒ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority ~~did not invite payment of any additional fee.~~ has therefore searched all groups that correspond to claims 1-35.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 11-87)